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A STATISTICAL STUDY OF THE POSSIBLE RELATIONSHIP BETWEEN CANCER OF THE STOMACH AND SOIL.

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VARIOUS studies (Diehl and Tromp, 1954) on the geographical distribution of cancer (all sites together) in the Netherlands suggest a soil-cancer relationship. In these studies also a historical review was given of similar observations in Great Britain by A. Haviland, A. T. Brand, H. T. Butlin, A. Jackson and C. D. Legon; and in France by Fiessinger, Foucault, Geuillot, Mollière, Robinet, Delbet and others. In connection with a recent field study in Wales, by the British Empire Cancer Campaign under leadership of Dr. Percy Stocks, Davies and Wynne Griffith (1954*a*) and Wynne Griffith and Davies (1954) were able to establish a statistically significant relationship between type of soil in the County of Anglesey and mortality frequencies of cancer of the stomach.

In Table I, for the period 1900–1940, a summary is given of the average cancer mortality (all sites together) per decade, per 100,000 inhabitants (both sexes together) for 7 principal soil units in the Netherlands. These mortality data were obtained as follows: the municipalities in the Netherlands were grouped together according to the type of soil on which the larger part of those municipalities (smallest administrative geographical unit in the Netherlands) was located. This classification was carried out with assistance of the Agricultural Soil Survey Institute at Wageningen. The total number of cancer deaths (all sites together) in each of these municipal groups during the period 1900–1940 (and each of the four decades separately) was standardized on 100,000 inhabitants (males and females together) above the age of 50 years.

Table I indicates that in each decade separately and for the average period 1900–1930 and 1900–1940 almost the same ranking by cancer mortality is found. If for each soil unit the percentage of " plus " municipalities is determined (*i.e.* the ratio between the number of municipalities located on a soil unit with average cancer mortality above the average of the country as a whole and the total number of municipalities belonging to that same soil unit), the succession is even more consistent and is statistically significant (see Appendix No. 1).

One could object that, despite the standardization on the population above 50,

the observed differences are due to differences in age group structure of the population above the age of 50, living on the various soil units. However, a detailed analysis was carried out (Diehl and Tromp, 1954) which does not support this viewpoint. For example on sea clay soils, with an average cancer mortality (and percentage " plus " municipalities) lower than that of the reclaimed peat soils, the percentage of population above 65 years of age on the total population is 7·2 against 6·8 on the reclaimed peat soils, and 6·3 on the peat soils. Other soil-cancer analyses support the observation that high average cancer mortality (or percentage of " plus " municipalities) based on the population over 50 cannot be explained in most instances by high percentages of population over 65 and *vice versa*.

More convincing proof is obtained by comparing, for one particular cancer site, the average cancer mortality per soil unit and for the different 5 year age groups, in males and females separately (Table II). In that case the observed differences could not be explained either by differences in age structure, cancer site frequency or sex.

Of the various cancer sites the stomach was selected because in case a soil-cancer relationship exists it might well show up most clearly in cancer of the stomach considering the relationship soil-vegetation-food.

TABLE I.—*Cancer Mortality on Various Soil Units in the Netherlands* 1900–1940.

Soil unit.	Number of municipalities (and of people living) on soil unit (in 1930).	Cancer mortality (all sites) per 100,000 inhabitants > 50 years (living on soil unit) and % " plus " municipalities (between brackets). 1900–1909.	1910–1919.	1920–1929.	1930–1939.	Average, 1900–1930.	Average, 1900–1940.	Percentage rural (R) and urban (M) municipalities.
Reclaimed peat soils	11 (118,953)	658 (82)	706 (82)	661 (55)	689 (82)	675	678	R = 20 U = 0
Peat soils	70 (322,760)	607 (63)	667 (60)	667 (53)	688 (57)	647	657	R = 83 U = 2
Sea-clay soils	275 (923,204)	595 (56)	654 (54)	667 (50)	693 (58)	639	652	R = 91 U = 0·5
Sandy soils (all types together)	385 (2,568,629)	562 (48)	622 (49)	653 (50)	651 (55)	612	622	Varying
Cover-sand soils	191 (1,013,762)	544 (43)	611 (47)	653 (50)	603 (49)	603	603	R = 82 U = 2
River-clay soils	193 (723,928)	551 (43)	603 (40)	635 (46)	631 (41)	596	605	R = 90 U = 3

Rural municipalities: municipalities with <5,000 inhabitants.
Urban " " " >20,000 "

For explanation of cover-sand soils see Table II.

Reliability of Mortality Data in the Netherlands.

Holland is a very favourable area for detailed geographical-pathological studies.

(1) Registration of deaths is accurate.

(2) Secrecy of death certificates is assured, so there is no hesitation of the part of the physicians to indicate the real cause of death.
(3) The mortality data are collected by the Netherlands Central Bureau of Statistics, a highly competent institution.
(4) Except in the largest cities, the population does not migrate very much. In the smaller agricultural municipalities migration can practically be neglected.
(5) Physicians from the medical state universities in the Netherlands, with similar educational facilities, are spread all over the country and students from one particular university do not concentrate after graduation in particular areas.
(6) All over the country well-equipped hospitals occur with modern X-ray and other facilities.
(7) In the smaller municipalities the population lives on the local food growing in the neighbourhood and local animals are used for meat consumption.

Factors Obscuring the Soil-Cancer Relationship.

As many important factors affect the cancer death rate it is evident that strong variations in those factors may obscure the soil-cancer relationship. For this reason a perfect correlation cannot be expected particularly if different decades are compared. Some of the main disturbing factors can be summarized as follows :

(1) Differences in intensity of other factors affecting the stomach-cancer mortality differing in the various soil groups and for different decades.
(2) Increase of the carcinoma death rate (for various reasons) during the period 1900–1940, the rate varying for the different municipalities on the various soil units.
(3) Changing character and different growing speed of the various municipalities changing the social, professional and other conditions.
(4) Consumption of vegetable food grown on soils different from those surrounding the municipalities.
(5) Local differences in acidity, moisture content in the same type of soil which may change considerably the local chemical balance of soils; *e.g.* a soil normally provided with magnesium may act locally as a magnesium deficient soil if the soil is very acidic as a result of seasonal flooding by rivers. Poor drainage and water logging often mobilizes the fixed phosphorus in soil and increases the availability to plants. It also affects the amount of organic compounds in soil and may cause certain trace element deficiencies.
(6) Differences in climate during different decades: *e.g.* in the province of Zeeland manganese deficiency diseases occur in plants after very long dry periods, especially on soils rich in humus, the influence being different for different types of soil and during different decades.
(7) Differences in exploitation of soil during different decades: *e.g.* considerable changes occurred in the chemical balance of soil in the Netherlands during the last 80 years because of changes in drainage, heavy manuring, ploughing up of grassland, reclamation of peat and heath areas, etc.

Cancer of the Stomach in the Netherlands in Relation to Soil.

For the period 1946–1952 two calculations were carried out :

(1) The total number of deaths from cancer of the stomach in men above the age of 50 in municipalities located on a particular soil unit, were related to the population of 10,000 men above the age of 50 and the percentage of " plus " municipalities was determined (see above). Except for cover sands, the same succession was found as indicated in Table I (particularly the decade 1930–1939): reclaimed peat soils: 80 per cent; peat soils: 61 per cent; sea clay soils : 64 per cent; sandy soils: (all types together): 58 per cent; river clay soils: 50 per cent; cover sands: 68 per cent.

(2) A similar calculation was carried out for each of the 5 year age groups above 50 in order to exclude any possible influence of differences in age group structure. The results are compiled in Table II.

TABLE II.—*Stomach Cancer Mortality on Various Soil Units.*

Soil unit.	Number of deaths from cancer of the stomach per 10,000 of the male population living on the various soil units (during the 7-year period 1946–1952) in the following age groups							Total number of deaths in men, above 50 years, from cancer of the stomach, during the 7-year period 1946–1952.
	50–54 (1895)	55–59 (1890)	60–64 (1885)	65–69 (1880)	70–74 (1875)	75–79 (1870)	80+ (1865)	
Reclaimed peat soils	55	82	137	189	359	482	648	255
Sea-clay soils	36	72	121	202	318	470	643	2969
Peat soils	44	83	107	258	553	439	600	433
Cover sands	45	69	121	198	357	465	559	2013
Sandy soils (all types)	39	67	110	186	325	436	553	4991
Ordinary sandy soils	33	65	99	176	302	407	522	2190
River-clay soils	32	51	104	167	251	414	467	1360
Average for the country	35	64	109	188	304	443	567	Total for country 14,072

(1) Numbers between brackets in the age group columns indicate the approximate year of birth of this particular age group.
(2) Cover sands : rather fine, well sorted sands with grain sizes particularly between 105 and 210 mμ, deposited by wind action at the end of the Würmglacial period.
(3) Ordinary sandy soils : sandy soils usually as a result of heath reclamation.
(4) The total of 14,072 includes deaths on soil units not mentioned in Table II because the total population living on those soil units is very small.

Table II warrants the following conclusions :

(1) In all age groups the river clay soils have a lower average stomach cancer mortality than the seaclay soils. The same relationship holds for the sandy soils (all types), peat soils and reclaimed peat soils as compared with the riverclay soils.

(2) The ordinary sandy soils have higher average stomach cancer mortality than the river-clay soils in 5 of the 7 age groups ; the reclaimed peat soils, peat soils and sea-clay soils have higher rates than the ordinary sandy soils in all age groups.

(3) The cover sands have higher average stomach cancer mortality rates than the ordinary sandy soils in all age groups.

(4) A comparison between the other groups indicates that in 4 to 6 of the 7 age groups the succession in stomach cancer rates remains the same.

In order to establish whether, despite these irregularities, the correlations can be considered to be significant, a mathematical study was carried out by Dr. C. A. G. Nass, Head of the Department of Medical Statistics of the Netherlands Institute of Preventive Medicine. I wish to express my gratitude for his assistance in this matter. The result of the analysis is compiled in Appendix No. 2. The final conclusion can be summarized as follows: a true association between stomach cancer mortality and types of soil is highly probable on the basis of a " variance test " using ranked mortalities. A mathematical " sign " test supports this statement.

(5*a*) In all age groups (except one, in case of the sandy soils) the river-clay soils and ordinary sandy soils have average stomach cancer mortalities below the average of the country and can be classified as " minus soils " For the oldest age groups it is true for all sandy soils (also the cover sands).

(*b*) seaclay, peat and reclaimed peat soils are " plus soils " in all age groups.

(6) The increase in stomach cancer mortality with increasing age is very great after the age of 65. The ratios between the stomach cancer mortality in the age group 80 and older and the group 50–54 is highest for the sea clay soils (17·8), followed by river-clay (14·6), sandy soil (all types : 14·2), peat soil (13·6), reclaimed peat soil (11·8). The same succession is found for the ratio of the age group 75–79 and 50–54. In other words, except for the seaclay soils, one has the impression that the increase in the oldest age groups is inverse with the average mortality, *i.e.* greatest increase in soils with lowest average stomach cancer mortality.

Possible Causes of the Soil-Stomach Cancer Relationship.

We have seen that differences in age group or sex could not explain the observed relationship. Still there are a number of factors which could be responsible for such a relationship : differences in racial and genetic factors (and blood groups), differences in type, character and growing speed of municipalities on the various soil groups.

The geographical distribution maps of cancer of the stomach were discussed with experts of the Department of Anthropogenetics, of the Netherlands Institute of Preventive Medicine. No obvious correlations were found between soil units and racial or genetic groups in the Netherlands. Blood group data in the Netherlands are insufficient for such a comparative study. In view of the observation of Aird and Bentall (1953) that cancer of the stomach seems to be more frequent amongst people of Blood Group A and particularly rare in Group O, it is worth while to study in future this possible cause of the observed correlation.

Differences in type and character of municipalities cannot explain the observed relationship because, as indicated in Table I, sea-clay soils and river clay soils have almost the same percentage of rural municipalities (90 per cent). In fact we do not know of any factor which could give a reasonable explanation of the observed

correlation. Therefore we are inclined to believe that the relationship, at least partly, is a true causal relationship. We do not like to enter into any speculative hypotheses at the present stage of research. However, we should like to point out only that if certain trace element distributions in soil, drinking water and food would affect the origin and (or) development of cancer of the stomach, for example by disturbing the enzyme balance in the body cells (as suggested by the studies of van Everdingen (1952) and others) or in counteracting the glycolitic and other processes in cells (as suggested by the studies of Hecht and Eichholtz (1929) and others), the soil-cancer relationship could be more easily understood.* In Table III a summary is given of the known distribution of a number of trace elements in the Netherlands. Differences in precipitation in different years, differences in drainage, acidity, lime, potassium, phosphate and iron content, etc., may lower or increase the expected available quantities as indicated in Table III. It is well known that several cattle diseases in the Netherlands and in other countries are closely related to soils with certain trace element deficiencies (or surpluses as in the case of Mo). Therefore it seems reasonable to assume that also "man" may be affected by certain trace element distributions. For iodine and fluorine it is even a generally accepted fact. If this would be true for other elements too, it seems possible that the observed soil-cancer relationship may prove to be, at least partly, a soil-trace element-cancer relationship. The second author, together with Dr. Lehr (Director of the Plant Nutrition Research Laboratory at Wageningen), is planning to carry out further research on this subject in the near future.

TABLE III.—*Distribution of Trace Elements in Soils in the Netherlands.*

Copper.	Cobalt.	Boron.
Little known about actual Cu contents of soils in the Netherlands 1. Sea-clay River-clay 2. Reclaimed heath-soils (ordinary sandy soils)	1. Peat soils Sea-clay River-clay 2. Sandy soils	1. Sea-clay 2. River-clay 3. Reclaimed peat soils 4. Sandy soils (*a*) If rich in organic matter often richer in Boron. (*b*) High acid and high lime content increase Boron deficiency

Iodine.	Magnesium.	Manganese.	Molybdenum.
1. Sea-clay 2. Peat soils 3. River-clay 4. Sandy soils	1. Sea-clay 2. Peat soils 3. River-clay 4. Sandy soils High acid and potassium content decreases available amount of MgO (*e.g.* flooded areas)	1. River-clay 2. Peat soils 3. Sea-clay 4. Sandy soils Reclaimed Peat soils Low acid, high P and Fe, high $CaCO_3$ and humus content lowers available amount of Mn	Surplus of Mo causes disease. 1. Light sea-clay (Wieringermeer) 2. Peat soils (Z.H.) 3. Frisian Sea-clay 4. Sandy soils Poorly drainage, alcaline soils and high Co content increases available amount of Mo

Numbers 1–4 indicate that the trace element content per kg. soil decreases from Group 1 to 4 (trace element deficiency).

* According to Hecht and Eichholtz (1929) particularly copper, but also zinc and bismuth seem to affect glycolitic processes in tumour cells.

Appendix 1 (*a*).

Mathematical Study of the Mortality Data of Table I.

(By the Department of Medical Statistics of the Netherlands Institute of Preventive Medicine at Leiden, Holland.)

For each decade the mortality figures of Table I can be ranked as indicated in Table IV.

Table IV.

Soil unit.	Ranked mortality figures. 1900–1909.	1910–1919.	1920–1929.	1930–1939.	Totals.
Reclaimed peat soils	1	1	3	2	7
Peat soils	2	2	1·5	3	8·5
Sea-clay soils	3	3	1·5	1	8·5
Sandy soils (all types)	4	4	4	4	16
River-clay soils	5	5	5	5	20
Totals	15	15	15	15	60

Analysis of variance :

Source.	Sums of squares.	Degree of freedom.	Mean squares.
Period	—	3	—
Types of soil	32·375	4	8·094
Error	7·125	12	0·594
	39·500	19	

$F = 8·094/0·594 = 13·6$

With 4 and 12 degrees of freedom the 0·1 per cent singificance level of F is 9·63, so the association between cancer mortality and types of soil is significant.

Appendix 1 (*b*).

Mathematical Study of the Percentages " Plus " Municipalities of Table I.

(By the Department of Medical Statistics of the Netherlands Institute of Preventive Medicine at Leiden, Holland.)

For each decade the percentages " plus " municipalities can be ranked as indicated in Table V and a " variance analysis " can be applied on these figures.

Table V.

Soil unit.	Ranked percentages " plus " municipalities. 1900–1909.	1910–1919.	1920–1929.	1930–1939.	Totals.
Reclaimed peat soils	1	1	1	1	4
Peat soils	2	2	2	3	9
Sea-clay soils	3	3	3·5	2	11·5
Sandy soils (all types)	4	4	3·5	4	15·5
River-clay soils	5	5	5	5	20
Totals	15	15	15	15	60

Analysis of variance :

Source.	Sums of squares.	Degree of freedom.	Mean squares.
Period	—	3	—
Types of soil	37·375	4	9·344
Error	2·125	12	0·177
	39·500	19	

$F = 9·344/0·177 = 52·8.$

With 4 and 12 degrees of freedom the 0·1 per cent significance level of F is only 9·63, so the association between percentages " plus " cancer municipalities and types of soil is highly significant.

Appendix 2.

Mathematical Study of the Mortality Data of Table No. 2.

(By the Department of Medical Statistics of the Netherlands Institute of Preventive Medicine at Leiden, Holland.)

The variance of the mortality figures clearly depends on the age groups. In the age group 50–54 it is apparently smaller than in the age group 80 and more. This fact prevents the immediate application of a " two-ways analysis of variance." The difficulty can be removed by ranking the mortality figures for each age group separately a indicated in Table VI.

Table VI.

	Ranked mortality figures according to age groups.							
Soil unit.	50–54.	55–59.	60–64.	65–69.	70–74.	75–79.	80 and more.	Totals.
Reclaimed peat soils	1	2	1	4	1	1	1	11
Sea-clay soils	4	3	2	2	4	2	2	19
Peat soils	3	1	4	1	3	4	3	19
Cover sands	2	4	3	3	2	3	4	21
Ordinary sandy soils	5	5	6	5	5	6	5	37
River-clay soils	6	6	5	6	6	5	6	40
Totals	21	21	21	21	21	21	21	147

Note : The group Sandy soils (all types together) was left out purposely because the mortality figures consist mainly of the group Cover sands and ordinary sands.

Analysis of variance :

Source.	Sums of squares.	Degree of freedom.	Mean squares.
Age groups	—	6	—
Types of soil	93·1	5	18·6
Error	29·4	30	0·980
	122·5	41	

$F = 18·6/0·980 = 18·98.$

With 5 and 30 degrees of freedom the 0·1 per cent significance level of F is only 5·12, so a true association between stomach cancer mortality and types of soil is highly probable.

Pairwise comparison of the types of soil is possible with the " sign test ". With only 7 pairs, significance at the 5 per cent level requires that all mortality figures of one type of soil are higher than the corresponding mortality figure of another type of soil.

The figures of Table II indicate that :

(1) the mortality figures of riverclay and ordinary sandy soils are significantly lower than those of other types of soil ;
(2) the mortality figures of cover sands are significantly lower than those of the reclaimed peat soils.

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INCIDENCE OF CARCINOMA OF THE CERVIX IN JEWISH WOMEN IN ISRAEL.

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THE low incidence of carcinoma of the uterine cervix among Jewish women was first noted by Braithwaite (1901). Since then many confirmatory reports have appeared (Fishberg, 1902; Auerbach, 1908; Theilhaber, 1909; Theilhaber and Greischer, 1910; Theilhaber, 1910; Sanders, 1916; Vineberg, 1919; Horwitz, 1927; Sorsby, 1931; Peller, 1931; Hoffman, 1933; Weir and Little, 1934; Smith, 1931; Davidsohn, 1939; Kaplan and Rosh, 1947). Kennaway (1948) has given an excellent critical review of the literature.

No one has been able to explain the remarkably low incidence of this common type of easily diagnosed cancer among Jewish women, which might be due to genetic factors or environmental ones, or to habits of hygiene.

Racially the Jewish people are far from uniform; their communities in countries all over the five continents who acknowledge themselves as Jews show a great variety of physical types. The three main groups (Ruppin, 1930–31) or communities, which we distinguish to-day, are descended from the Jews of ancient Judea, who were exiled to Babylonia (Iraq). Those who remained there or migrated to the neighbouring countries such as Syria and Persia and as far as Egypt, Yemen and North Africa belong to the Mizrahi or Oriental community. The descendants of those who migrated to Spain and Portugal and were exiled at the end of the fifteenth century, belong to the Sephardic community. They are found chiefly in the countries bordering the Mediterranean. The third group, the Ashkenasi Jews, consists of the descendants of those who migrated to Eastern Europe before the Middle Ages and then spread over Europe and migrated to Britain and America.

These three groups have lived for many centuries among different peoples and in very different milieus; some of their customs have changed, but their physical appearance has changed also. This was due probably to slow racial admixture despite interdiction of intermarriage, hence Jews of the different communities resemble one another no more than do the Nordic and Mediterranean races. Thus many northern European Jews are tall, light skinned, blue-eyed blonds, while the Yemenite Jew, for example, is usually short and slim, with dark brown skin and dark hair and eyes.

Since the beginning of this century, and particularly in the past decade, there has been a great return from all over the world to Israel (Palestine) of Jews, who have to a very large extent retained their group ties and their habits. Furthermore, they have not intermarried to any large extent. It is, therefore, of interest to determine whether the low incidence of carcinoma of the cervix, which has so far

been ascertained only for the Ashkenasi women, prevails also among the other Jewish groups, particularly those of the East.

Another inquiry to which the study of the cancer material of Israel lends itself is the relationship between the incidence of cancer of the uterine cervix and the observance of the Biblical Talmudic Law of sexual abstinence—Niddah—(Kennaway, 1948) for at least five days during the menses and for seven days thereafter. The Mosaic Law also prescribes abstinence from intercourse for forty days after the birth of a male and eighty days after the birth of a female. On the whole the proportion of those who observe these religious laws in Israel is less among the Ashkenasi than among the Mizrahi and Sephardi group. The great majority of Ashkenasi women abstain from sexual intercourse only during vaginal bleeding, as do many other non-Jewish people.

Another Jewish law, the circumcision of the male one week after birth, is observed by practically all Jews all over the world, and in Israel as well. Its probable influence on the incidence of cervical carcinoma will be discussed later.

MATERIAL.

All cases of cancer of the uterine cervix at the Rothschild Hadassah University Hospital, Jerusalem, between 1933–1951 are included in this study. The Radium Institute was opened in 1933 and for many years was the only place in this country where radium treatment was used. Since the gynaecologists of this country did not perform Wertheim operations, they referred all cases of cervix carcinoma to this Institute. Thus practically all cancers of the cervix occurring in Jewish women in Israel are included in our material.

This consists of 262 cases, of which 125 are Jewish, and the rest Moslem or Christian Arabs or Christians of European descent. Probably many cases among Arab women were not referred here for treatment for various reasons. Hence our material does not indicate the true incidence of this disease among the Arab population.

In the more recent material all diagnoses were based on histological examinations. Among the earlier cases the report of the biopsy was frequently not available. However, the clinical findings and the course of the disease were typical in all cases. Among the 125 Jewish cases were 56 squamous cell carcinomas and 11 adenocarcinomas of cervical origin. In the remainder the report of the histological examination was not available.

Owing to difficulty in distinguishing the records of Sephardi Jews, and those of the various sub-groups of Mizrahi Jews, all the non-Ashkenasi elements are grouped together under the title Mizrahi (Table I). The greatest number of cases in both classes occurred in the 45–54 age group.

TABLE I.—*Classification of Cases according to Age and Ethnic Groups.*

Ethnic group.	Age unknown.	Up to 34.	35–44.	45–54.	55–64.	Over 64.	Total.
Ashkenasi	1	11	18	19	18	12	79
Mizrahi	1	4	10	18	9	4	46
	2	15	28	37	27	16	125

Table II gives the incidence according to the histological and clinical findings in the two ethnic groups.

TABLE II.—*Histological and Clinical Findings in the Two Ethnic Groups.*

Ethnic group.	Squamous cell carcinoma.	Adeno-carcinoma.	On clinical evidence only.	Total.
Ashkenasi	36	8	35	79
Mizrahi	20	3	23	46
	56	11	58	125

Childbearing has been considered important in the etiology of cancer of the cervix. The classification of our cases by parity is given in Table III.

TABLE III.—*Number of cases of Carcinoma of the Cervix by Parity*, 1933–1951.

Number of children unknown	26
No children	18
One child	21
2–3 children	19
4 or more children	41
Total number of cases	125

Table IV states the parity of the Jewish married women over the age of 18 in Israel. (Figures are available only for the year 1951.)

TABLE IV.—*The Parity of Married Women in Israel above the Age of* 18 *in* 1951.

	%
Women without children	33·5
„ with one child	29·6
„ „ two children	21·8
„ „ three children	8·1
„ „ four and more children	7·0
	100·0

From the Central Bureau of Statistics and Economic Research, Israel.

Table V gives the average number of children per thousand inhabitants in Israel and in the other parts of the world during the years 1933–1951.

TABLE V.—*Birth Rate per* 1000 *Inhabitants* 1933–1951 *in*—

Israel	28·5
U.S.A.	20·3
U.K.	16·4
New Zealand	21·9

The data are taken from Statistical Abstracts of Israel (1953–54) of the Central Bureau of Statistics and Economic Research, Israel, and from the Demographic Year Book (1953) U.N.

We encountered great difficulties in establishing the incidence of the disease in each group, as during the period 1933–1951 the composition and number of the population was very variable. We had to collect data of the general population, as well as of every ethnic group, in every year.

The total Jewish population, the Jewish female population, the number of Ashkenasi and Mizrahi Jews for those years which we could ascertain, and the incidence of cancer of the cervix for the period under study are given in Table VI. There was an average of 2·2 cases for 100,000 Jewish women during the period

1933–1951. The difference of incidence in the two ethnic groups is not statistically significant.

The true ratio between cancer of the cervix and cancer of other sites of the female genitalia could not be established in our material, as for the period under discussion there are no statistical data available in this country. However, we will add the statistical material of the Hadassah University Hospital. For the same period the *hospital* incidence of cancer of the uterine body was 131 ; of cancer of the ovary 199 ; and of cancer of the breast 891, while the *total* incidence of carcinoma of the cervix was 125. Therefore, carcinoma of the body is more frequent than carcinoma of the cervix in the Jewish population of Israel. This is in sharp contrast to the statistical findings of other countries, where the ratio is just the reverse ; for example, the ratio of cervix to corpus carcinoma (Casper, 1954) in the United States, Canada, South Africa and Australia is 2·5 to 3·5 : 1 ; in Switzerland, Jugoslavia and Germany 4 : 1 to 7 : 1 and among Hindus and various ethnic groups of East Asia 40 : 1 to 50 : 1.

DISCUSSION.

On the basis of our material from Israel we could confirm again the relatively low incidence of carcinoma of the cervix in Jewish women. Although we included in our figures squamous-cell carcinoma and adenocarcinoma, as well as cases in which the diagnosis of carcinoma was made on clinical evidence only, the figures are still very low. We could not find in statistics dealing with the incidence of the cervix carcinoma in general and of Jewish women in particular whether any distinction has been made so far between the squamous cell type and the adenocarcinoma, although this difference may have a bearing on the etiological factors of the disease and the low incidence in Jewish women may be associated only with the squamous cell carcinoma. Thus, making no distinction between the different types, incidence remains low and is 2·2 per 100,000 Jewish women with relatively slight variations from year to year.

For comparison a few more figures may be mentioned : during the years 1943–1947 the incidence of cervical cancer in Copenhagen was 28 per 100,000 inhabitants per year (Clemmesen and Nielsen, 1951) and in Detroit (Cutler and Rowan, 1945) the incidence in 1948 was 20 per 100,000 inhabitants, and 39·3 per 100,000 female inhabitants.

The reverse relation between cancer of the cervix and cancer of the uterine body in comparison with other countries (Casper, 1954) has been shown and gives further evidence of the low incidence of the cervical carcinoma of the Jewish population in Israel.

The incidence is 1·1 per 100,000 Ashkenasi and 1·4 per 100,000 Mizrahi inhabitants (Table VI), two groups of very different ethnical typology. The equal incidence in both groups is clear evidence that the low incidence in Jewish women is not a racial phenomenon.

Possibly some of our clinically diagnosed cases are carcinomas of the uterine body. Yet, even with the inclusion of these cases, the incidence is exceedingly low and, according to our Table II, there is statistical equality of incidence of squamous cell carcinoma in the Ashkenasi and in the Mizrahi group, and the possibility of the aforementioned error cannot, therefore, invalidate our conclusion.

Childbearing has been considered to increase the incidence of cancer of the

TABLE VI.—*Incidence of Carcinoma of the Cervix among Ashkenasi and Mizrahi Jews from* 1933–1951.

Year.	Number of Jewish inhabitants.	Number of Ashkenasi Jews.	Number of Mizrahi Jews.	Number of Jewish women.	Carcinoma of Cervix. Number of cases in Ashkenasi Jews.	Number of cases in Mizrahi Jews.	Total number of cases.	Per 100,000 Ashkenasi.	Per 100,000 Mizrahi.	Per 100,000 women.
1933	235,000	—	—	115,000	—	—	1	—	—	—
1934	283,000	—	—	138,500	—	—	4	—	—	2·9
1935	355,000	—	—	176,000	—	—	4	—	—	2·3
1936	384,000	294,500	89,500	192,000	3	2	5	1	2·2	2·6
1937	396,000	—	—	198,500	—	—	3	—	—	1·5
1938	411,000	315,000	96,000	206,000	2	1	3	0·6	1·1	1·1
1939	445,500	—	—	221,500	—	—	5	—	—	2·3
1940	463,500	—	—	229,500	—	—	4	—	—	1·7
1941	474,000	—	—	234,000	—	—	8	—	—	3·4
1942	484,500	—	—	239,000	—	—	7	—	—	2·9
1943	508,500	399,000	103,500	247,500	4	1	5	1	1	2·0
1944	529,000	—	—	259,500	—	—	9	—	—	3·5
1945	579,000	450,000	129,000	282,000	8	2	10	1·9	1·6	3·5
1946	595,500	462,500	133,000	290,000	4	3	7	0·7	2·3	2·4
1947	630,000	488,000	142,000	305,500	4	2	6	0·8	1·4	2·0
1948	759,000	—	—	367,000	—	—	3	—	—	0·8
1949	1,014,000	702,500	311,500	491,000	10	2	12	1·4	0·6	2·5
1950	1,203,000	797,500	405,500	586,000	8	7	15	1·0	1·7	2·6
1951	1,404,500	871,000	533,500	689,500	11	4	15	1·3	0·7	2·2
					54	24	125			
					78					
					Average number of cases per 100,000 for each group			1·4	1·4	2·2

cervix and this was explained on grounds of repeated trauma. According to our material (Table III, IV) there seems to be a higher frequency of cervical carcinoma among women with a higher birth rate, for even looking at the few available statistical data with great caution, cervical cancer appears to be more frequent among the women with more children.

On the other hand (Table V) the birth rate in Israel is higher than in many other countries. Yet in spite of this the incidence of cervical cancer in Jewish women is very low.

Observance of the Niddah ritual has been thought by some authors (Cohen, 1949) to be the reason for the low incidence of cervical cancer. However, the majority of Ashkenasi women, though abstaining from sexual intercourse during menstruation, usually do not abstain during the succeeding seven days. The incidence of cancer of the cervix is low among them, but it is the same among the Mizrahi women, who observe the ritual to a greater extent. It would be very desirable to observe this point more closely in the future, and Jewish women with cancer of the cervix should be interrogated precisely on this question.

Another possible explanation of the low incidence of cervix carcinoma in Jewish women is the circumcision of the Jewish male. The low incidence of penile carcinoma among circumcised communities, Jews as well as Moslems, led many authors (Wolbarst, 1949; Bleich, 1950; Ravich and Ravich, 1951; Wynder, Cornfield, Schroff and Doraiswani, 1954) to believe that smegma is carcinogenic. Smegma of the non-circumcised male brought into contact during intercourse with the cervical epithelium may be one of the carcinogenic factors in the etiology of cancer of the cervix. In the whole cancer material of our Institute there was not a single case of penile carcinoma. In India cervical and penile carcinoma are much more frequent in the Hindu group not practicing circumcision than among the Moslem group that does circumcise, though both belong to the same ethnic group. Gagnon (1950) did not find a single case of cervix carcinoma among 3,280 nuns in Canada. The incidence of cervical cancer among Jewish women married to non-circumcised men would be of interest.

Experimentally Fishman *et al.* (1942) could not prove any carcinogenic properties of human smegma on the vaginal or cervical epithelium in mice. But later Plaut and Kohm-Speyer (1947) showed that horse smegma is carcinogenic to mice.

SUMMARY.

(1) The incidence of cancer of the uterine cervix in Jewish women is 2·2 per 100,000.

(2) The Jewish population of Israel is composed of communities of different ethnic typology, but the incidence of cancer of the uterine cervix in all these communities is of the same order. Hence the low incidence of cancer of the cervix in Jewish women cannot be dependent on a racial factor.

(3) The Niddah ritual does not seem to be the factor to which the low incidence of cervical carcinoma can be attributed, but an enquiry on this matter in individual cases of cervical carcinoma in Jewish women is very desirable.

(4) Circumcision of the Jewish male seems to be the explanation for the low incidence of cervical carcinoma. The absence of smegma due to circumcision is probably the most important preventing factor of cervical cancer in Jewish women.

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INTESTINAL METAPLASIA OF THE GASTRIC MUCOSA.

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DURING the routine histological examination of gastrectomy specimens removed for benign as well as malignant conditions it is not uncommon to find areas of intestinal epithelium in the gastric mucosa. The presence in the stomach lining of this type of epithelium has, in the past, been regarded as a congenital abnormality (Hari, 1901 ; Borrmann, 1926 ; Taylor, 1927 ; and Clar, 1934). However, the majority of observers in recent years favour the view that the intestinal epithelium seen in the gastric mucosa is due to a process of metaplasia in post-foetal life (Geissendorfer, 1928 ; Faber, 1935 ; Magnus, 1937 ; Stout, 1943 ; Schindler, 1947). They believe, in the words of Magnus, that " the presence of intestinal epithelium in the stomach is the result of faulty regeneration of surface epithelium in a mucosa repeatedly damaged by gastritis, and is an example of metaplasia resulting from chronic irritation" . The importance of intestinal metaplasia has been pointed out by Warren and Meissner (1944) who regard it as the chief epithelial feature of chronic gastritis.

The purpose of this paper is to draw attention to the existence of intestinal metaplasia and to describe its incidence and extent in the mucosa of stomachs removed surgically for duodenal ulcer, gastric ulcer and carcinoma.

MATERIAL AND METHODS.

All the material used in this investigation consists of specimens removed at operations for duodenal ulcer, gastric ulcer and carcinoma. They are subdivided as follows:

Duodenal ulcer . . .	52	specimens.
Gastric ulcer	28	,,
Carcinoma of the stomach .	39	,,
Total	119	

Strips of stomach wall, about one centimetre wide, were taken from the entire length of the lesser and greater curvatures of every specimen ; the pylorus was included in each strip. These varied from 5 to 10 inches long. They were rolled up like a " swiss roll " (Magnus, 1937) and secured in position by a fine thread tied around the circumference. In some cases it was convenient to dissect the mucosa off the submucosa and roll up the mucous membrane by itself. By this method very long strips of gastric mucosa can be examined in one section.

Staining techniques.—(1) Haematoxylin and eosin. All sections were stained by this method, using Ehrlich's acid haematoxylin. Normal gastric mucus is not stained at all by this method, but the goblet cell mucus characteristic of intestinal epithelium is stained blue. (2) Southgate's modification of Mayer's

muci-carmine stain. This method was only used to confirm the presence of goblet cell mucus which it stains red. It does not stain normal gastric mucus. (3) The Diazo method for staining argentaffin granules (Jacobsen, 1939). The granules are stained by Brentamine Fast red B salt (Imperial Chemical Industries). The argentaffin granules appear rust red against a yellow background. This method is quick and reliable.

On the basis of differences in the glands of which the gastric mucosa is composed the stomach may be divided for descriptive purposes into four topographical " sites ". These are, (1) the pylorus, (2) the lesser curve, (3) the greater curve, (4) the fundus.

The pylorus.—The pylorus is that part of the stomach which contains the pyloric glands. It may be called the pyloric " antrum " or the pyloric " region ", but for the purposes of this study it is referred to as the " pylorus ". It occupies approximately the distal quarter of the whole stomach. It is easily identified in the sections by its characteristic glands, but care must be taken not to confuse them with the areas of pseudo-pyloric metaplasia that often occur in the region of the body and fundus of the stomach. Even if intestinal metaplasia is very extensive, there is usually some evidence of the remains of typical pyloric mucosa. The pylorus is included in the " swiss roll " sections that were taken from the entire length of both curvatures of the stomach.

The lesser curve.—Anatomically this extends from the cardia to the pyloro-duodenal junction on the right border of the stomach. However, for the purposes of this study it is defined as the part of the anatomical lesser curve which contains the gastric glands. Its junction with pyloric mucosa is not sharply defined and the mucous membrane of the one mixes with that of the other over a short distance in the region of the incisura angularis.

The greater curve.—Anatomically this extends from the cardia to the pyloro-duodenal junction on the left border of the stomach. However, for the purpose of this study it is defined as that part of the greater curve which contains the gastric glands, other than the fundus. It extends somewhat further towards the pylorus than the lesser curve and almost reaches the pyloro-duodenal junction.

The fundus.—The fundus extends from the cardia to a point on the greater curve marked off by a plane passing horizontally through the cardiac orifice. It is only present in specimens in which total gastrectomy was performed.

" *Extent* " *of intestinal metaplasia.*—This records the amount seen within each one of the topographical sites, and is graded as follows :

Intestinal metaplasia, Grade I	= +	or slight extent.
,, ,, ,, II	= + +	or moderate extent.
,, ,, ,, III	= + + +	or very extensive.

Grade I is awarded when metaplasia is present, but only in the form of scattered islands that form a very small part of the total area under consideration. However, if just one area of microscopic size is seen this grading is given, for often the process is confined to a group of three or four gastric tubules only.

Grade II is given when the islands of intestinal metaplasia have become confluent and form larger areas of metaplasia involving not more than half the total area under consideration.

Grade III intestinal metaplasia is awarded when more than half the total area under consideration is affected. Sometimes it is complete throughout the site and very little evidence of normal gastric mucosa can be found.

THE IDENTIFICATION OF INTESTINAL METAPLASIA. (Fig. 1–3)

The striking feature that distinguishes intestinal metaplasia from normal gastric epithelium is the goblet cell. This contains a droplet of mucus that is stained blue by Ehrlich's haematoxylin and red by Southgate's modification of Mayer's muci-carmine method. According to Järvi and Lauren (1951) there are two types of mucus in the human alimentary tract—gastric and intestinal. Only mucus of intestinal type stains with muci-carmine. The normal gastric mucosa is lined by tall, very regular, simple columnar epithelium and contains no goblet cells of this type. If goblet cells are seen in the gastric mucosa it can be inferred that intestinal type epithelium is present (Magnus, 1937). The surface cells of the metaplastic epithelium are columnar in type with a striated border. This feature is not seen in normal gastric epithelium but is characteristic of epithelial cells in the intestine. The goblet cells are scattered among the columnar cells and vary in number from about one goblet cell to every three or four epithelial cells up to a stage in which almost every cell in the mucosa contains the characteristic droplet of mucus. This has led, in the past, to the use of the term " goblet cell metaplasia " to describe these appearances. The deeper parts of the tubules in intestinal metaplasia are lined by columnar epithelium containing scattered goblet cells, but their nuclei are larger and more darkly staining than their counterparts in normal gastric epithelium. Goblet cells tend to be fewer in the deeper parts of the mucosa. At the bases of the metaplastic gastric glands there are features that are entirely characteristic of intestinal epithelium and are rarely found, if at all, in the normal gastric mucosa. The first of these is the Paneth cell, often seen in large numbers in areas of intestinal metaplasia, and rarely difficult to find. They are low columnar cells with basal nuclei and cytoplasm packed with coarse granules that stain brightly with eosin. In between the Paneth cells at the bases of the gastric glands argentaffin cells may be seen. These are smaller than their neighbours, but have a relatively large oval nucleus with a characteristic chromatin network. The cytoplasm contains argentaffin granules that are basal in position with the nucleus lying between them and the lumen of the tubule. They can be seen with difficulty in ordinary haematoxylin and eosin preparations, but may be clearly demonstrated by the Diazo method.

The main histological features of intestinal metaplasia have been described. They resemble, in all respects, the features of the epithelium that is found in the intestine (Magnus, 1937). However, there is a closer resemblance to the lining epithelium of the large intestine than of the small bowel. The latter shows slender villi that are not characteristic of intestinal epithelium in the gastric mucosa and goblet cells are found in much greater numbers in the large intestine.

The extent of involvement of the gastric mucous membrane varies from the conversion of a single tubule to involvement of large areas of mucosa. Uncommonly the whole lining of the stomach may be affected and it is only possible to find evidence of its gastric origin by a careful search for specialised gastric epithelial cells, such as pyloric glands. The thickness of the areas of intestinal metaplasia varies greatly. It usually conforms to that of the adjacent epithelium. In atrophic gastritis it is the same thickness as the rest of the gastric epithelium of which it is a part. However, intestinal metaplasia is not only found in association with chronic atrophic gastritis, but may also be present in other forms of gastritis. Also, in pernicious anaemia much or all of the body mucosa may be replaced by intestinal epithelium in the absence of any inflammatory lesion (Magnus, 1952).

Specialised cells of the body of the stomach may survive the process of metaplasia, and although reduced in number they are not difficult to find in most cases. On the other hand, in those cases in which large areas of the stomach mucous membrane are involved, the peptic and oxyntic cells will be entirely absent. The pyloric glands are affected in the same way, but it is usually possible to identify a particular region of the gastric mucosa that is metaplastic by surviving cells of characteristically pyloric or body type.

THE INCIDENCE AND EXTENT OF INTESTINAL METAPLASIA

Total incidence.—A total of 119 stomachs removed for duodenal ulcer, gastric ulcer and carcinoma were examined for the presence of areas of intestinal metaplasia with the following result :

Metaplasia present in 93 out of 119 cases	=	78·2%
„ absent in 26 „ „ „ „	=	21·8%
		100·0%

These figures show that in the examination of stomachs removed surgically one would expect to find intestinal metaplasia in the great majority of them, provided that a sufficient area of the gastric mucosa is examined ; for it must be emphasised that a positive observation includes cases in which only a very small amount of metaplasia can be seen.

Incidence within the three groups of stomachs (Table I).—An examination of Table I shows how the incidence of intestinal metaplasia varies within the three groups of stomachs under consideration. In stomachs containing carcinoma the incidence is 92·3 per cent, in the gastric ulcer group it is 82·1 per cent, and in the duodenal ulcer cases it is 65·4 per cent. It can be stated, therefore, that the incidence of intestinal metaplasia is greatest in cases of carcinoma and least in cases of duodenal ulcer, with cases of gastric ulcer taking an intermediate position. This table also emphasises the difference between stomachs containing carcinoma on the one hand and the combined non-cancerous group on the other ; it shows that there is, in fact, a greater incidence of intestinal metaplasia in cancerous than non-cancerous stomachs.

TABLE I.—*Incidence Within the Groups.*

Group.	− VE.	+ VE.	Total.	% + VE.	Non-cancerous. − VE.	Non-cancerous. + VE.	Non-cancerous. Total.	Non-cancerous. % + VE.
D.U.	18	34	52	65·4				
G.U.	5	23	28	82·1	23	57	80	*71·25*
Cancer	3	36	39	*92·3*				

Incidence at the sites (Table II).—In the stomachs removed for duodenal ulcer the pylorus is involved in nearly 60 per cent of cases, whereas the lesser curve (15·4 per cent) and the greater curve (9·6 per cent) are seldom affected. The gastric ulcer series is somewhat different ; in these the incidence at the lesser curve (57·1 per cent) and the greater curve (39·3 per cent) is relatively higher, although the pylorus still maintains its position as the site most often affected (71·4 per

cent). In the gastric cancer group the order of incidence, pylorus, lesser curve and greater curve still holds good, but there is a more even distribution between the three sites. The reason for this lies in the fact, as will be shown later, that a very high proportion of cases of carcinoma shows involvement by intestinal metaplasia at all three sites (Table IIIa). It can be stated, then, that the incidence of intestinal metaplasia is greatest at the pylorus, followed by the lesser curve and greater curve in that order, whichever group of stomachs is considered.

TABLE II.—*Incidence at the Sites.*

Site.	D.U. (%).	G.U. (%).	Carcinoma (%).
Pylorus	59·6	71·4	87·1
Lesser curve	15·4	57·1	79·5
Greater curve	9·6	39·3	66·7

The distribution of positive observations by group and site.—Table IIIa shows that in stomachs removed for duodenal ulcer involvement of one site only is the rule (24 out of 34 cases containing intestinal metaplasia) and Table IIIb shows that this site is the pylorus. In Table IIIc it is apparent that when two-site involvement occurs in duodenal ulcer it is the combination of pylorus with lesser curve that is the most frequently affected. This combination of sites is known as the gastric canal or " magenstrasse ".

In stomachs removed for gastric ulcer two-site involvement is the most common (10 out of 23 cases containing intestinal metaplasia, Table IIIa), and Table IIIc shows that it is the combination of pylorus with lesser curve (or gastric canal) which is the most frequently affected. However, when one site only is considered (Table IIIb) it is always the pylorus which is involved. It should be

TABLE IIIa.—*Distribution of Positive Observations by Group and Site.*

Group.	Total.	+ VE	Present in: One site only.	Two sites only.	All three sites.
D.U.	52	34	24	10	0
G.U.	28	23	6	10	7
Carcinoma	39	36	5	7	24

TABLE IIIb.—*One-site Involvement.*

Site.	D.U.	G.U.	Carcinoma.	Total.
Pylorus	21	6	3	30
Lesser curve	2	0	1	3
Greater curve	1	0	1	2
Totals	24	6	5	35

TABLE IIIc.—*Two-site involvement.*

Site.	D.U.	G.U.	Carcinoma.	Total.
Pylorus and lesser curve	6	6	6	18
Pylorus and greater curve	4	1	1	6
Lesser curve and greater curve	0	3	0	3
Totals	10	10	7	27

noted that there is a relatively high proportion of cases of gastric ulcer in which all three sites are affected.

In cancerous stomachs the results show the exact opposite to those found for duodenal ulcer; namely, that three-site involvement is the most common (24 out of 36 cases containing intestinal metaplasia, Table IIIa). However, if one site only is involved it is usually the pylorus (Table IIIb), and when two-site involvement is considered it is nearly always the combination of pylorus with lesser curve (or gastric canal) which is affected.

It is the consideration of two-site involvement which is important. Apart from " pylorus with lesser curve " there are two other possible combinations of two-site involvement which can occur. They are " pylorus with greater curve " and " lesser curve with greater curve" . Table IIIc shows that these are not often found in any of the three groups of stomachs under consideration. Thus, out of a total of 119 stomachs examined, 27 show two-site involvement only, and of these the " pylorus with lesser curve " combination is found in 18 or 66·6 per cent (Table IIIc). When it is also remembered that it is nearly always the pylorus that is affected in one-site involvement (30 out of a total of 35 cases in all groups, Table IIIb) it can be seen that the part of the stomach most frequently affected by intestinal metaplasia is the gastric canal or ' magenstrasse '.

The extent of intestinal metaplasia. (Table IV).—In the great majority of cases of duodenal ulcer Grade I observations were made (24·4 per cent); these were mostly found at the pylorus (26 out of 38 Grade I observations). Only very occasionally were Grade II (2·6 per cent) or Grade III (1·3 per cent) observations made, and all of these except one were made at the pylorus. The only Grade II observation not at the pylorus is affecting the lesser curve.

In stomachs removed for gastric ulcer most of the observations are Grade I (34·5 per cent), but unlike the stomachs removed for duodenal ulcer these are more evenly distributed among the three sites. However, the order pylorus, lesser curve and greater curve is still present. The Grade II observations (7·1 per cent) are greater than in duodenal ulcer and they, too, are distributed more evenly among the three sites in the same descending order of extent. The striking feature, however, in cases of gastric ulcer is the relatively high proportion of Grade III observations (14·3 per cent) when compared with duodenal ulcer (1·3 per cent). These observations, which represent the most extensive intestinal metaplasia seen in gastric ulcer, are found most frequently in the region of the pylorus, and least frequently on the greater curve, with the lesser curve taking an intermediate position.

In the cancerous stomachs observations of all three grades are more evenly distributed among the three sites than in either gastric or duodenal ulcer. There are almost as many Grade III observations (26·5 per cent) as there are Grade I, (30·8 per cent). Also, it can be stated that the descending order of frequency for site, already demonstrated, is still the same. When cancerous stomachs are compared for extent of intestinal metaplasia with stomachs removed for duodenal and gastric ulcer the significant feature is the much greater proportion of Grade II and Grade III observations in the cancerous group. There are nearly three times as many Grade II observations in carcinomatous stomachs as there are in gastric ulcer specimens, and nearly ten times as many as there are in stomachs removed for duodenal ulcer. The greater extent of intestinal metaplasia in cancerous stomachs is emphasized by the fact that there are nearly twice the number of

Grade III observations in cancerous stomachs as in cases of gastric ulcer and more than twenty times the number in specimens removed for duodenal ulcer.

It can be stated, then, that the extent of intestinal metaplasia is greatest in stomachs containing carcinoma and least in those with duodenal ulcer, with cases of gastric ulcer taking an intermediate position. Further, the site most extensively affected by intestinal metaplasia in all cases is the pylorus, with the lesser and greater curves following in that order. However, in cases of duodenal ulcer intestinal metaplasia is mostly confined to the pylorus, and is seldom very extensive. In gastric ulcer it is greatest at the pylorus, and least on the greater curve, with the lesser curve taking an intermediate position. In cancerous stomachs, although the same descending order of frequency is still present, intestinal metaplasia is more evenly distributed over the three sites.

TABLE IVa.—*Extent of Intestinal Metaplasia by Group and Site. Duodenal Ulcer.*

Extent.	Pylorus.	Lesser curve.	Greater curve.	Total.	Per cent.
0	21	44	47	112	71·7
+	26	7	5	38	24·4
++	3	1	0	4	2·6
+++	2	0	0	2	1·3

Total number of stomachs = 52
" " " observations = 156

TABLE IVb.—*Extent of Intestinal Metaplasia by Group and Site. Gastric Ulcer.*

Extent.	Pylorus.	Lesser curve.	Greater curve.	Total.	Per cent.
0	8	12	17	37	44·1
+	11	10	8	29	34·5
++	3	2	1	6	7·1
+++	6	4	2	12	14·3

Total number of stomachs = 28
" " " observations = 84

TABLE IVc.—*Extent of Intestinal Metaplasia by Group and Site. Carcinoma.*

Extent.	Pylorus.	Lesser curve.	Greater curve.	Total.	Per cent.
0	5	8	13	26	22·2
+	12	13	11	36	30·8
++	8	7	9	24	20·5
+++	14	11	6	31	26·5

Total number of stomachs = 39
" " " observations = 117

The points system (Table V).—Another, and in many ways more convincing, method of demonstrating the extent of intestinal metaplasia in the three groups of stomachs is by the use of a points system. In this each stomach is awarded one point for every + observation made. For example if metaplasia is absent, obviously no points are given to that stomach; but if a Grade II (++) observation has been made at the pylorus, and there is no metaplasia elsewhere in the

stomach, then two points are awarded. This means that a maximum of nine points can be earned by a stomach which has Grade III (+++) intestinal metaplasia for each of the three sites under consideration.

The results are given in Table V. Under each group the number of stomachs are given for which a particular number of points has been awarded. The total number of points in each group are then divided by the number of stomachs. This gives the mean number of points per stomach. The results show that the mean number for carcinoma is 4·5, for gastric ulcer 2·8 and for duodenal ulcer 1·0. If the cancerous group of stomachs is then compared with the combined non-cancerous groups the figures show that the stomachs removed for carcinoma (mean number = 4·5) have more than twice the amount of intestinal metaplasia than those removed for gastric and duodenal ulcer (mean number = 1·9).

The points system confirms (1) that the amount of intestinal metaplasia is greatest in stomachs containing carcinoma and least in those with duodenal ulcer, with the gastric ulcer group taking an intermediate position, (2) that intestinal metaplasia is more extensive in cancerous than non-cancerous stomachs.

TABLE V.—*Points System.*

	D.U.	G.U.	Carcinoma.
Total stomachs in each group	52	28	39
Total points per group	52	77	177
Mean number of points/stomach	1·0	2·8	*4·5*
Non-cancerous series combined		129 points	
Mean number of points/stomach		*1·9*	

The incidence and extent of intestinal metaplasia at the fundus of the stomach.—This is considered separately as observations made on the fundus can only be recorded in cases of total gastrectomy for carcinoma. In addition to the 39 stomachs previously considered a further 24 cases of total gastrectomy were available for a study of the fundus, making a total of 63 cases. Intestinal metaplasia was present in 20, or 31·8 per cent. This is the least incidence of any of the sites previously considered. In other words, in stomachs containing carcinoma there is less intestinal metaplasia at the fundus than at the pylorus, lesser curve or greater curve.

Of the 20 cases or 31·8 per cent in which intestinal metaplasia was found the extent was graded as follows :

Intestinal metaplasia absent				in 43 cases.	
,,	,,	Grade	I	,, 16	,,
,,	,,	,,	II	,, 4	,,
,,	,,	,,	III	,, 0	,,
				63	

EXPLANATION OF PLATES.

FIG. 1.—Normal pyloric mucosa. Haematoxylin and eosin. × 50.

FIG. 2.—Normal body mucosa. Haematoxylin and eosin. × 50.

FIG. 3.—Intestinal metaplasia of gastric mucosa. Note numerous goblet cells and contrast with the remains of three tubules lined by normal superficial gastric epithelium. Haematoxylin and eosin. × 50.

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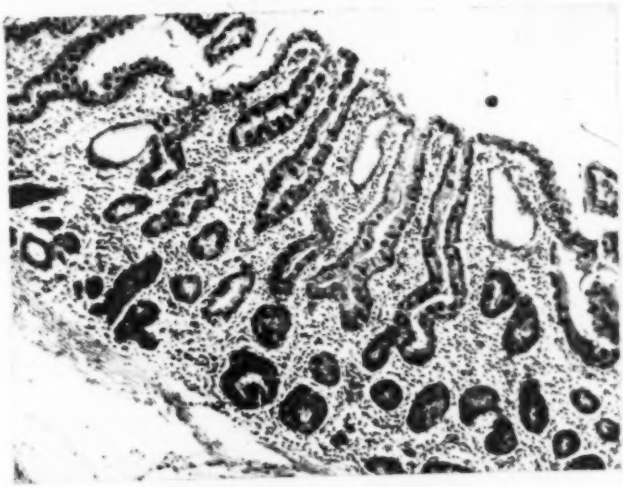

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Grade III intestinal metaplasia was never seen and in the majority of cases in which metaplasia was found Grade I was awarded. This shows that in stomachs containing carcinoma the extent as well as the incidence of metaplasia at the fundus is less than at any of the other sites.

Effect of age and sex.—The age and sex of the patients from which the gastrectomy specimens were removed could not be obtained in all cases. Out of the 119 stomachs studied the age and sex for 89 are available.

In Table VI all the 89 cases available are distributed in five age categories and the average number of points per stomach (see points system, Table V) is given for each category. The results show that the extent of intestinal metaplasia gradually increases with age.

TABLE VI.—*Effect of Age.*

All groups.	Number of cases.	Number of points.	Average.
Under 35	10	5	0·5
35–44	14	37	2·6
45–54	24	67	2·8
55–64	21	70	3·3
65 +	20	78	3·9
	89	257	

An analysis is now made of the sex incidence in 89 of the specimens of duodenal ulcer, gastric ulcer and carcinoma examined for incidence and extent of intestinal metaplasia. The sex incidence in all three groups is given below and conforms to that generally accepted.

	Male.	Female.
Duodenal ulcer	26	3
Gastric ulcer	17	9
Carcinoma	22	12
	65	24

If the points system is used for comparing the extent of intestinal metaplasia in the two sexes, the following result is obtained :

24 females obtain 64 points—2·7

65 males obtain 190 points Average—2·9

This result suggests that the amount of intestinal metaplasia found in gastrectomy specimens is not affected by consideration of the sex of the patient.

DISCUSSION.

It has been pointed out by Magnus (1937) that the frequency with which intestinal metaplasia is found in the stomach depends to a great extent upon the amount of gastric mucosa examined. In the routine laboratory examination of gastrectomy specimens only a very short length of mucous membrane in the immediate vicinity of ulcer or carcinoma is usually sectioned. The rest of the stomach lining is seldom investigated unless it appears macroscopically abnormal. This probably explains why the existence of intestinal metaplasia is not well known, and gets only the briefest mention in text-books of pathology.

Magnus (1937) using the " swiss roll " technique found intestinal metaplasia in 59 of his 100 partial gastrectomy specimens. In this study it has been shown that 78·2 per cent of 119 gastrectomy specimens contain areas of intestinal meta-

plasia. Stout (1945) examined a series of 150 stomachs equally divided between duodenal ulcer, gastric ulcer and carcinoma. Intestinal metaplasia was present in nearly 70 per cent of all cases, but he does not give details of the amount of mucosa examined in each stomach. Puhl (1926) found intestinal epithelium in every one of his 140 specimens of gastric ulcer. But, Heyrovsky (1912) found it in only 14·2 per cent of cases removed for " simple ulcer " and Taylor (1927) in 11 per cent of specimens of gastric ulcer. However, Taylor admits that his figure is probably far below the true incidence. The discrepancy between these results may be explained by the different amount of mucosa examined by the various observers. It would appear, anyway, that the presence of intestinal epithelium in the gastric mucosa is a common enough finding provided large areas of the gastric mucous membrane are examined.

It has been shown that the incidence and extent of intestinal metaplasia is greatest in stomachs containing carcinoma and least in those with duodenal ulcer, with cases of gastric ulcer taking an intermediate position. Also, that cancerous stomachs show a greater incidence and extent than the combined non-cancerous group. This confirms the work of Stout (1945). Geissendorfer (1928) has described the topographical distribution of intestinal metaplasia as being in the descending order of frequency, pylorus, lesser curve, greater curve and fundus. Chuma (1923) and Borrmann (1926) state that intestinal metaplasia is most frequently found in the pyloric part of the stomach. The results of this study confirms these findings both for the incidence and extent of intestinal metaplasia.

The fundus of the stomach is not removed in operations for duodenal ulcer or gastric ulcer, and has therefore not been studied in these two groups of specimens. However, in specimens of total gastrectomy for carcinoma it has been shown that the fundus shows less intestinal metaplasia than any of the other three sites examined. In none of the 63 cases examined was it ever very extensive. In view of this finding and the fact that the distribution of intestinal metaplasia in the duodenal ulcer and gastric ulcer groups is mostly found in the pyloric and lesser curve regions of the stomach, it can be safely presumed that the fundus is never extensively involved by intestinal metaplasia. Cases of pernicious anaemia may be an exception to this (Magnus, 1952).

There does not seem to be any investigation in the literature on how the topographical distribution of intestinal metaplasia varies within the three groups of stomachs, duodenal ulcer, gastric ulcer and carcinoma. In duodenal ulcer intestinal metaplasia is usually found at the pylorus only, and is seldom severe. However, when the other sites are considered the lesser curve is affected more frequently than the greater curve. Further, when two-site involvement is considered it is the combination of " pylorus and lesser curve " which is most frequently affected. Involvement of all three sites does not occur at all in duodenal ulcer in this series. In gastric ulcer metaplasia involves the three sites in the order, pylorus, lesser curve and greater curve. However, when two-site involvement is considered it is still the combination of " pylorus and lesser curve " which is most frequently affected. In stomachs containing carcinoma the incidence and extent of intestinal metaplasia decreases in the order, pylorus, lesser curve and greater curve, but the difference between the three sites is not very great. The reason for this is that the cancerous stomachs show a very high proportion of cases which are involved at all three sites. However, when two-site involvement only is found, it is nearly always the combination of " pylorus with lesser curve "

which is affected. It would appear, then, that whichever group of stomachs is considered it is the pylorus and the combination of " pylorus with lesser curve " which are the sites most frequently and extensively affected by intestinal metaplasia. This part of the stomach is known as the gastric canal or magenstrasse. Pillenkamp (1929) points out that the region of the greater curvature is the most important part of the stomach for the secretion of the gastric juice and that the more essential function of the gastric canal is that of a passage. It follows that the gastric canal will be more exposed to injury than the rest of the stomach. It is also well known that gastric disease, whether benign or malignant, more frequently affects the region of the pylorus and lesser curve than any other part of the stomach. With these facts in mind, it is easy to understand why the mucous membrane of the gastric canal more frequently undergoes intestinal metaplasia.

The replacement of gastric by intestinal epithelium may be very extensive. Magnus (1937) reports two cases of gastric ulcer in which the entire stomach was replaced by intestinal epithelium, only a few areas of degenerate gastric mucosa remaining. One of the gastric ulcer cases in this series and five of the cancerous stomachs showed almost complete replacement by intestinal epithelium, but the fundus still contained evidence of ordinary, but atrophic, gastric mucosa.

The quantitative assessment of the extent of intestinal metaplasia shows that cancerous stomachs contain more than four times the amount seen in specimens removed for duodenal ulcer, and nearly twice the amount seen in cases of gastric ulcer. These results should be considered in the light of Stout's (1945) findings, confirmed in this study, which suggest that the amount of intestinal metaplasia increases with the succeeding decades of life. This suggestion was first made by Geissendorfer (1928). Faber (1935) regards extensive replacement of the gastric mucosa by intestinal epithelium as being particularly common in old persons. The average age of patients with duodenal ulcer is lower than in those with gastric ulcer or carcinoma, so that if age is a factor in the development of intestinal metaplasia one would expect cases of duodenal ulcer to show less of it. Similarly one would expect cancerous stomachs to show more metaplasia than cases of gastric ulcer, for the mean age of patients with carcinoma of the stomach is higher than those with gastric ulcer.

If age is a factor in the development of intestinal metaplasia, then it is important to know whether it affects the conclusion that cancerous stomachs contain more intestinal metaplasia than non-cancerous stomachs. The mean age of patients with carcinoma in this series is 59·9 years, with gastric ulcer 52·8 years and duodenal ulcer 44·3 years. From these figures it can be seen that the cancerous stomachs were removed from individuals of older age groups than in the combined non-cancerous series. In order to neutralize the age factor to some extent Stout (1945) has compared the amount of intestinal metaplasia in cancerous and non-cancerous stomachs in the sixth decade and in those over 59 years of age. In both of these, allowing for age, there is still more intestinal metaplasia in the cancerous than the non-cancerous stomachs. This method of neutralizing the age factor may be repeated by using the points system (Table V) already used for studying the effect of age.

	Age.	Number. Points.	Number. Cases.	Average.
Cancerous stomachs	50–59	27	6	4·5
Non-cancerous stomachs	50–59	29	15	1·9
Cancerous stomachs	60 +	108	21	5·1
Non-cancerous stomachs	60 +	17	11	1·6

If, then, it is accepted that the amount of intestinal metaplasia increases with advancing years the above results confirm Stout's (1945) figures, which show that, allowing for the age factor, stomachs containing carcinoma show more intestinal metaplasia than those removed for benign gastric and duodenal ulcer.

SUMMARY AND CONCLUSIONS.

The incidence and extent of intestinal metaplasia of the gastric mucosa has been investigated and the appearances described.

The results show that intestinal metaplasia is a common finding provided large areas of the gastric mucosa are examined. Its incidence and extent is greatest in stomachs removed for carcinoma and least in those with duodenal ulcer, with cases of gastric ulcer taking an intermediate position. Further, the incidence and extent of intestinal metaplasia is greater in cancerous than non-cancerous stomachs.

In stomachs removed for duodenal ulcer intestinal metaplasia is mostly confined to the pylorus. It is seldom extensive. In stomachs removed for gastric ulcer the incidence and extent of intestinal metaplasia is greatest at the pylorus and least on the greater curve with the lesser curve taking an intermediate position. In stomachs removed for carcinoma intestinal metaplasia is distributed more evenly over the three sites than in duodenal or gastric ulcer, but it is still greatest at the pylorus, followed by the lesser and greater curves in that order.

An analysis of the incidence and extent of intestinal metaplasia in all three groups of stomachs taken individually and as a whole, shows that the part of the stomach most frequently and extensively affected by intestinal metaplasia is the combination of pylorus and lesser curve or the gastric canal.

I am indebted to Professor H. A. Magnus and Dr. F. Avery Jones for advice and encouragement, also to the many surgeons who have kindly provided me with gastrectomy specimens. Expenses have been defrayed out of a block grant by the British Empire Cancer Campaign.

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CARCINOMA ARISING FROM AREAS OF INTESTINAL METAPLASIA IN THE GASTRIC MUCOSA

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It has been demonstrated that there is more intestinal metaplasia of the gastric mucosa in cancerous than non-cancerous stomachs (Stout, 1945 : Morson, 1955). Also, that very large areas of the gastric mucous membrane may be replaced by epithelium of intestinal type. In view of these findings it is suggested that some cases of gastric carcinoma may arise from areas of intestinal metaplasia. Histological evidence in support of this is presented in the following pages.

MATERIAL AND METHODS.

1. The primary growth and the mucous membrane in its vicinity have been examined in 107 gastrectomy specimens removed for carcinoma of the stomach. Every one of these specimens had at least 2 and often as many as 6 blocks of tissue taken from the primary growth. All of them were cut from the edge of the primary carcinoma and include a strip of the adjacent mucous membrane. In this way any transition from metaplastic mucosa to carcinoma may be detected.

2. In addition to pieces of tissue from the primary growth " swiss roll " sections of the gastric mucosa (Magnus, 1937 ; Morson, 1955) were available in the search for areas of early malignant change. The appearances of pre-invasive carcinoma or carcinoma *in situ* are valuable evidence of the histogenesis of malignant tumours. The absence of invasion answers the criticism that the appearances may have been produced by infiltration of the mucous membrane from without.

All sections were stained with haematoxylin and eosin, and with Southgates' modification of Mayers' muci-carmine for confirming the presence of intestinal mucus.

RESULTS.

Five examples of the origin of gastric carcinoma from areas of intestinal metaplasia are described and illustrated with microphotographs. In the first of these a gastrectomy specimen is described which contains a very early carcinoma apparently arising from an intestinal type of epithelium. In Examples 2 and 3 the transition from metaplastic mucosa to carcinoma at the edge of a primary tumour is demonstrated. In neither of these cases is there any invasion of the submucosa at the point of transition, and the histological appearances do not suggest invasion of the mucous membrane from without. In the last two examples the appearances of pre-invasive carcinoma or carcinoma *in situ* arising in epithelium of intestinal type are described. These two examples were found in gastrectomy specimens removed for carcinoma, but at a distance from the main tumour. They may be regarded as independent, primary foci of malignancy. There was no

indication of the presence of these areas of pre-invasive carcinoma during naked-eye examination of the stomachs concerned. Both of them were found accidentally during the histological examination of " swiss-roll " sections of the gastric mucosa.

Goblet cells are the most striking characteristic of intestinal epithelium, and are only found in the stomach in association with areas of intestinal metaplasia (Magnus, 1937). They contain a droplet of mucus which stains blue with Ehrlich's haematoxylin and red with Southgate's modification of Mayer's muc-icarmine. According to Järvi and Lauren (1951) there are two types of mucus in the alimentary tract—gastric and intestinal. Only intestinal or goblet cell mucus is stained by muci-carmine. All the examples described in the following pages has been examined for the presence of this intestinal type of mucus. It is suggested that the presence of this in a carcinoma of the stomach is evidence of its origin from an intestinal type of epithelium.

Example No. 1.

Description of specimen.—Total gastrectomy with attached spleen and greater omentum. On the anterior wall of the pyloric antrum about 2 inches from the pyloro-duodenal junction there is a plaque of papillary growth 1 inch in diameter. No other macroscopic abnormality seen.

Histology (Fig. 1–3).—Sections show a mucoid adeno-carcinoma with early invasion of the submucous tissues of the stomach wall (Fig. 1). The whole of the mucous membrane lining this stomach shows, with the exception of the fundus, complete replacement by an intestinal type of epithelium (Fig. 3). The fundus shows only a slight degree of intestinal metaplasia.

This is an early carcinoma which is secreting mucus with the staining properties of the intestinal type and not the gastric variety. It is histologically very similar to adenocarcinomas of the lower bowel and rectum, and there is continuity between metaplastic mucosa and carcinoma at the edge of the tumour (Fig. 1 and 2). The mucous membrane surrounding the carcinoma is completely metaplastic (Fig. 3). There is no indication of the presence of any normal gastric mucosa and the appearances could quite easily be mistaken for intestinal or rectal mucous membrane.

In view of the extensive replacement of the lining of this stomach by epithelium of intestinal type, the nature of the mucus secreted by the carcinoma, and its likeness to adenocarcinomas of the rectum and colon, it is suggested that it is arising from an area of intestinal metaplasia and not from ordinary gastric mucosa.

Example No. 2.

Description of specimen.—Total gastrectomy with attached spleen and greater omentum. There is a hard plaque of growth involving the middle third of the lesser curvature. It is about 2 inches in diameter and lies $1\frac{1}{2}$ inches from the cardia and the same distance from the pyloro-duodenal junction. No other macroscopic abnormality seen.

Histology. (Fig. 4–6).—Sections show a moderately well-differentiated, mucus-secreting adenocarcinoma. Long strips of mucous membrane from the region of the pylorus, lesser curve, and greater curve show very extensive areas of intestinal metaplasia. There is no metaplasia at the fundus of the stomach.

This tumour is very similar in appearance to adenocarcinomas of the large intestine and rectum. It is secreting mucus which stains blue with Ehrlich's

haematoxylin and red with muci-carmine. This suggests an origin from an intestinal type of epithelium. Sections taken from the edge of the tumour show complete replacement of the adjacent mucosa by an epithelium of intestinal type (Fig. 4). There is also a transition from metaplastic mucosa to carcinoma at this point. As there is no invasion of the muscularis mucosae or the submucosa where the transition takes place it is justifiable to assume that the malignant change is occurring in the metaplastic mucosa and the appearances are not those of invasion from without.

A study of the histological appearances at the point of transition reveals the following changes (Fig. 5 and 6). Passing from the metaplastic mucosa into carcinoma the tubules lose their regular outline. The cells lining them have become reduplicated and their nuclei are large and hyperchromatic. Many mitoses may be seen. At many points the hyperplastic cells have broken down the limiting membrane of the tubules and are invading the stroma. In other words the tissue has all the characteristics of malignant transformation. At the same time it contains the characteristics of an intestinal type of epithelium. Numerous goblet cells are present and occasional Paneth cells may be seen at the bases of the tubules. Moreover, there is a complete absence of any of the features of ordinary gastric mucosa.

In view of the extensive replacement of the lining of this stomach by epithelium of intestinal type, the nature of the mucus secreted by the tumour, and the histological evidence of a transition from metaplastic mucosa to carcinoma at the edge of the primary growth, it would appear that this carcinoma is arising from an area of intestinal metaplasia and not from ordinary gastric mucosa.

Example No. 3.

Description of specimen.—Partial gastrectomy with attached greater omentum. There is a nodular growth involving the entire circumference of the pylorus for a length of 1 inch. It extends right up to the pyloro-duodenal junction, but there is no invasion of the duodenum. No other macroscopic abnormality seen in the stomach.

Histology. (Fig. 7 and 8).—Sections show a well-differentiated adenocarcinoma invading the stomach wall and peri-gastric tissues. It is secreting a little mucus. Strips of mucous membrane from the region of the pylorus and body of the stomach show extensive areas of intestinal metaplasia.

This tumour is indistinguishable from those found in the large intestine and rectum. It is secreting mucus which stains blue with Ehrlich's haematoxylin and red with muci-carmine. This suggests an origin from an intestinal type of epithelium. Sections taken from the edge of the tumour show complete replacement of the adjacent mucous membrane by epithelium of intestinal type. At this point there is a transition from metaplastic mucosa to carcinoma (Fig. 7). As there is no invasion of the submucosa where the transition takes place it can be presumed that the appearances are those of malignant change *in situ* and not invasion from without.

A study of the histological appearances at the point of transition in this case reveals the following changes (Fig. 8). Passing from the metaplastic mucosa into carcinoma there is a gradual loss of differentiation. The tubules have become distorted in shape and size, and many of them are solid with proliferating cells.

Others are disintegrating and their cells are invading the surrounding stroma. The cells lining the tubules contain large, hyperchromatic nuclei and many mitoses can be seen. These are the characteristics of malignant change. The microphotographs (Fig. 7 and 8) show the likeness between the metaplastic mucosa and the carcinoma. Further, there appears to be a gradual transition from the one to the other. The metaplastic mucosa contains numerous goblet cells which gradually disappear as one passes further into the carcinomatous tissue. A number of Paneth cells are present. There is very little evidence of the presence of any ordinary gastric mucosa.

This stomach shows extensive replacement of its mucosa by an intestinal type of epithelium and its carcinoma is secreting mucus of intestinal type. Furthermore, there is a transition from metaplastic mucosa to carcinoma at the edge of the primary growth. In view of these findings it would appear that this carcinoma is arising from an area of intestinal metaplasia in the gastric mucosa.

EXPLANATION OF PLATES.

FIG. 1.—Example 1. Early adenocarcinoma of the stomach with invasion of submucous tissues. Haematoxylin and eosin. × 10.

FIG. 2.—Example 1. A higher power view of the junction between carcinoma and metaplastic mucosa at the extreme right-hand edge of the previous figure. Note the numerous goblet cells in the metaplastic mucosa. No gastric-type epithelium can be seen. Haematoxylin and eosin. × 50.

FIG. 3.—Example 1. Intestinal type of mucosa in neighbourhood of tumour. Note goblet cells. At the bases of the tubules there are numerous Paneth cells which appear very dark in the photograph. No normal gastric mucosa present. Haematoxylin and eosin. × 50.

FIG. 4.—Example 2. Junction of carcinoma with surrounding mucous membrane. Metaplastic mucosa on right. Note numerous goblet cells and absence of ordinary gastric mucosa. Invasive carcinoma at bottom left-hand corner of the photograph. There appears to be a transition from metaplastic mucosa to carcinoma. Haematoxylin and eosin. × 50.

FIG. 5.—Example 2. Higher power view of long tubule at left centre of Fig. 4. The appearances are those of an intestinal type of epithelium undergoing malignant change. Haematoxylin and eosin. × 100.

FIG. 6.—Example 2. High power view of left centre of previous figure. The characteristics of carcinoma are superimposed upon an intestinal type of epithelium containing numerous goblet cells. Haematoxylin and eosin. × 400.

FIG. 7.—Example 3. Junction of metaplastic mucosa and carcinoma at the edge of the primary tumour. There is continuity between the benign and malignant tissue. Invasion of submucosa at bottom left-hand corner. Haematoxylin and eosin. × 50.

FIG. 8.—Example 3. Higher power view of lower limit of previous figure. The metaplastic mucosa on the right shows numerous goblet cells and absence of characteristic gastric-type epithelium. Passing to the left of the photograph the metaplastic mucosa appears to be undergoing malignant transformation. Haematoxylin and eosin. × 100.

FIG. 9.—Example 4. Carcinoma *in situ*. The characteristics of malignant change are superimposed upon epithelium of intestinal type. No normal gastric mucosa is present. Haematoxylin and eosin. × 100.

FIG. 10.—Example 4. High power view of bottom right-hand corner of previous photograph. Note goblet cells and obvious carcinoma with early invasion of the stroma. Haematoxylin and eosin. × 450.

FIG. 11.—Example 4. Another view of area of carcinoma *in situ*. There is no invasion of the submucosa and the characteristics of intestinal epithelium and carcinoma are present. Haematoxylin and eosin. × 100.

FIG. 12.—Example 5. Carcinoma *in situ*. Numerous goblet cells are present, and the tubules are of intestinal type. The tubule at the left lower margin of the photograph is reminiscent of a normal pyloric gland. At a number of points the metaplastic tubules appear to be undergoing malignant change with invasion of the mucosal stroma. Haematoxylin and eosin. × 100.

FIG. 13.—Example 5. High power view of left centre of previous figure. Tubules lined by goblet cell epithelium are disintegrating and invading the surrounding stroma. Haematoxylin and eosin. × 450.

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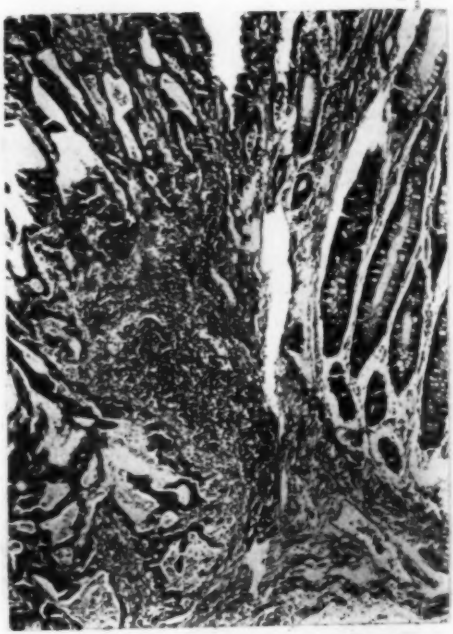

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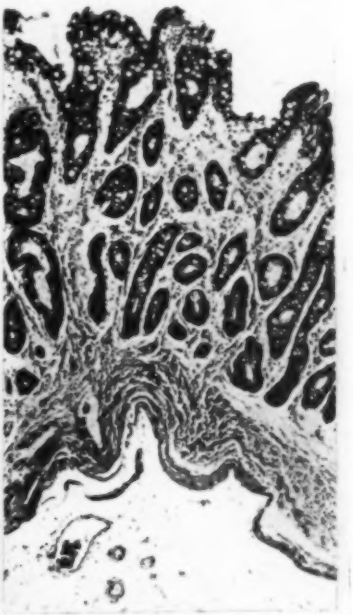

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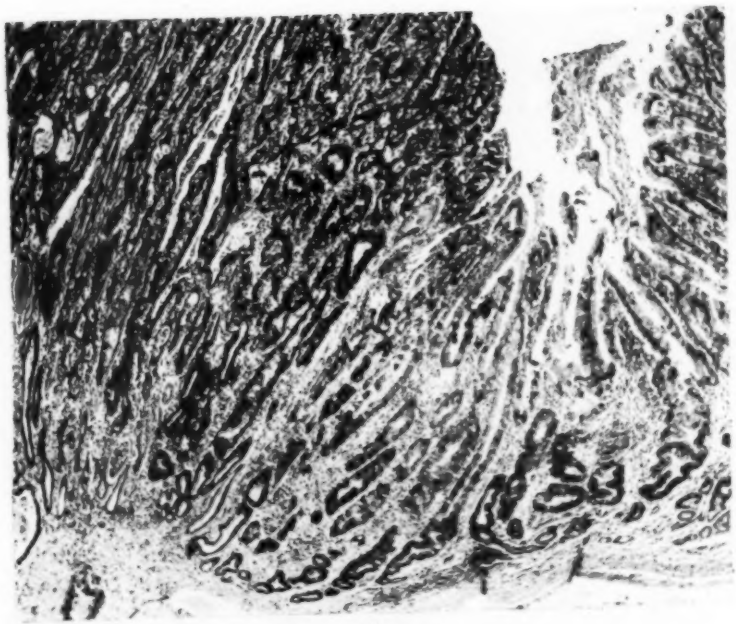

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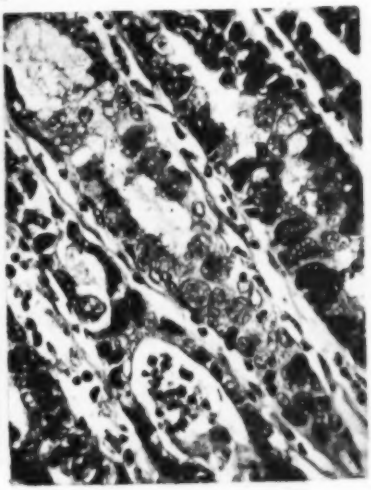

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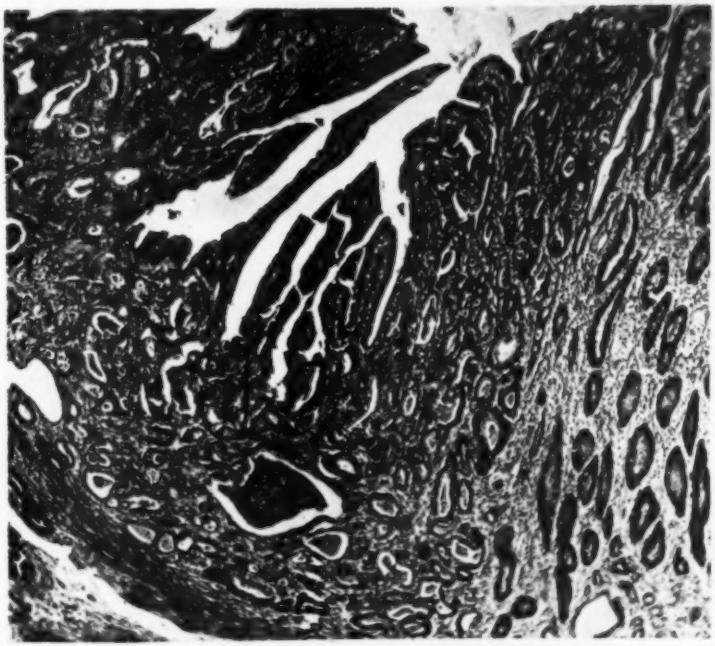

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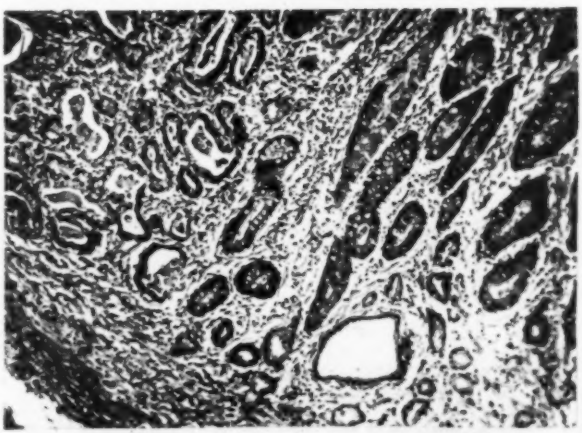

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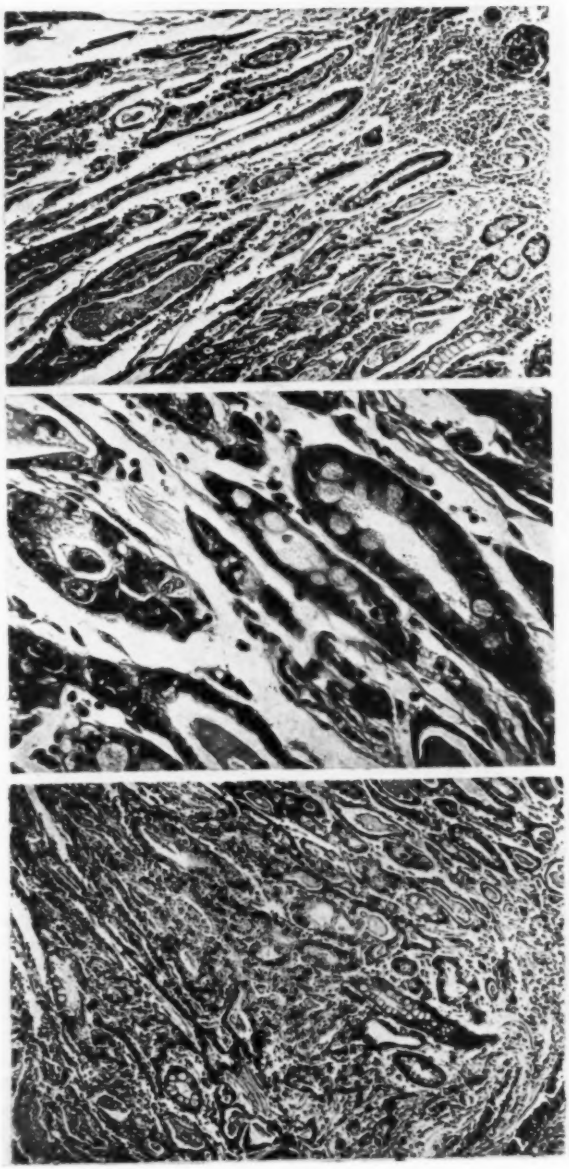

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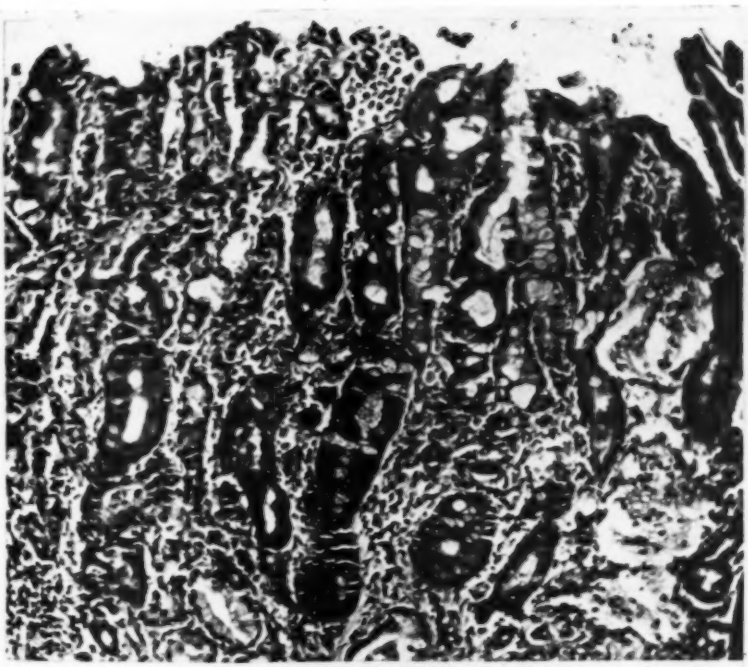

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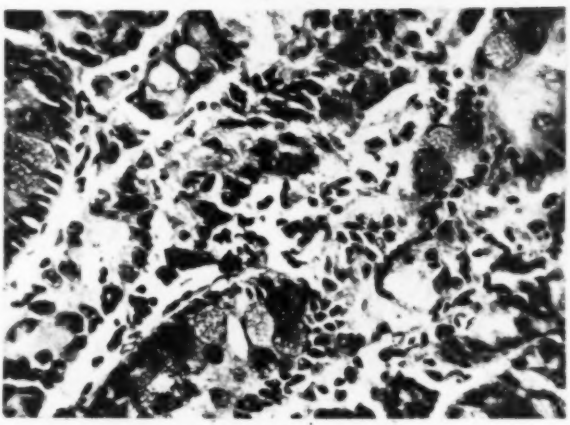

13

Example No. 4. (Fig. 9, 10, and 11).

During the examination of a " swiss roll " section of gastric mucosa from the posterior wall of one of the stomachs in this series an example of pre-invasive carcinoma was found. This appears to be arising in epithelium of intestinal type without invasion of the submucous tissues. It is involving a strip of mucous membrane about ½–1 inch long and is surrounded by mucosa which shows complete intestinal metaplasia. Little evidence of normal gastric mucosa can be seen, and there is a complete absence of characteristic gastric glands.

This area of pre-invasive carcinoma shows the features of intestinal epithelium as well as those of carcinoma. The tubules contain numerous goblet cells, which give the characteristic staining reactions for intestinal as opposed to gastric mucus. They are lined by cells containing large, hyperchromatic nuclei (Fig. 10) which show great variation in size and shape. Numerous mitoses are present. The tubules appear to be losing their differentiation, and are disintegrating to form a mass of carcinomatous cells (Fig. 9 and 11) which are invading the mucosal stroma (Fig. 10). There is no sign whatever of any ordinary gastric mucosa, and the malignant change appears to be superimposed upon a mucosa with the characteristics of an intestinal type of epithelium.

Example No. 5. (Fig. 12 and 13).

The examination of a " swiss roll " section containing a long strip of pyloric mucosa shows an area of intestinal metaplasia in which there are scattered foci of malignant change. There is hardly any evidence of the presence of ordinary gastric mucous membrane and the histological appearances suggest that the malignant change is superimposed upon epithelium of intestinal type.

The tubules are lines by columnar epithelium containing numerous goblet cells, which give the staining reactions for intestinal mucus. At the bases of the tubules the epithelium is very hyperplastic and many Paneth cells are present. Some of the tubules appear to be disintegrating, with loss of differentiation and invasion of the mucosal stroma by malignant cells. Numerous mitoses are present, some of them abnormal. A study of Fig. 12 gives a representative picture of this area of pre-invasive carcinoma. Relatively normal tubules are present which are of the intestinal type. They are mixed up with others which are definitely carcinomatous. In Fig. 13 a high power view is given which confirms the presence of goblet cells and invasion of the mucosal stroma by malignant cells. In Fig. 12 ragged mucous cysts can be seen. These are very commonly present when malignant change is taking place in an area of intestinal metaplasia, and they are probably due to the blocking of tubules by proliferating cells.

DISCUSSION.

Histologists of the early part of this century (Schmidt, 1896; Gossett and Masson, 1912; Anchutz and Konjetzny, 1921; Chuma, 1923) believed that some cases of gastric carcinoma arise from intestinal epithelium, and the evidence submitted in this paper supports their views. Stout (1945) failed to demonstrate the histological transition from intestinal metaplasia to carcinoma. But Warren and Meissner (1944) suggest that when the epithelial changes in intestinal metaplasia become severe they compare favourably with recognized pre-cancerous conditions found elsewhere in the body.

The evidence submitted in this paper is concerned only with the histological transition from intestinal metaplasia to carcinoma. There does not appear to be any investigation in the literature with which this evidence can be compared. However, it is interesting to contrast the histological appearances with those described and illustrated by Stewart and Lorenz (1947) in their article on the development of carcinoma from intestinal epithelium in mice treated with carcinogenic substances. This article, which is beautifully illustrated with microphotographs, gives the stages in the development of carcinoma from the epithelium lining the small intestine. The appearances are very similar to those described in this series for the transition from intestinal metaplasia of the gastric mucosa to carcinoma. But there is other evidence which supports the conclusion that gastric carcinoma may arise from epithelium of intestinal type.

There is an histological resemblance between gastric and intestinal carcinoma (Gossett and Masson, 1912). The majority of cases in both groups are adenocarcinomas of varying degrees of differentiation. In general, however, gastric cancers tend to be less well-differentiated than their counterparts in the intestine and rectum. There is also a much higher proportion of anaplastic malignant tumours among the gastric carcinomas. The similarity of gastric and intestinal cancer has led Mulligan and Rember (1954) to use the term " intestinal cell " to describe one of their three main histological types of gastric carcinoma. They classify carcinoma of the stomach into " mucous cell", " pyloro-cardiac " cell, and " intestinal cell " types. Out of their total of 138 cases they put 35, or about 25 per cent, in the " intestinal cell " group and trace their origin to areas of intestinal metaplasia in the gastric mucosa.

If some cases of gastric carcinoma arise from areas of intestinal metaplasia then they should contain evidence of the characteristics of intestinal epithelium. These include the secretion of goblet cell mucus and the presence of a striated border to the columnar cells lining the tubules. Neither of these characteristics is found in normal gastric mucosa and are only seen in the stomach when intestinal metaplasia is present. Järvi and Lauren (1951) investigated 184 specimens of gastric carcinoma and found histological evidence of a striated border to the carcinomatous cells in about 50 per cent of cases, even in the metastases. They reject previous theories that this is due to metaplasia within the tumour itself, and suggest that all gastric carcinomas which contain a striated border arise from areas of intestinal metaplasia in the gastric mucosa. They also investigated the characteristics of mucus in gastric cancers, and point out the difference between mucus of characteristically gastric type and that of intestinal type. In 30 per cent of their cases the mucus in the carcinomas was stained exclusively by muci-carmine, and is therefore of intestinal type. They also showed that in tumours containing a striated border, a mucous secretion of intestinal type was mostly observed. Järvi and Lauren (1951) conclude that a substantial proportion of gastric tumours originate from intestinal epithelium. It is a common observation to see mucus secretion in carcinomas of the stomach and Chuma (1923) suggests that the " signet ring " cells in colloid carcinomas are the malignant counterpart of the goblet cells seen in areas of intestinal metaplasia.

It is important to know what porportion of gastric carcinomas arise from areas of intestinal metaplasia. Of the 107 primary carcinomas examined in this study 35, or 32·7 per cent appear to be arising from epithelium of intestinal type. However, in some cases of carcinoma in this series it was evident that the tumour was

not arising from intestinal epithelium. A small proportion of the stomachs containing carcinoma showed no intestinal metaplasia at all. In others very little was seen. These points are mentioned because it is apparent that although a substantial proportion of gastric carcinomas may arise from areas of intestinal metaplasia, in many cases the malignant change occurs in other types of epithelium. It is conceivable that a carcinoma may arise from a solitary area of intestinal metaplasia which is completely destroyed by the expanding tumour. However, in the great majority of cases in which the origin of the primary growth from intestinal epithelium could be demonstrated there was very extensive intestinal metaplasia of the rest of the gastric mucosa. Mulligan and Rember (1954), place about 25 per cent of gastric carcinomas in their "intestinal cell" group. Järvi and Lauren (1951) have shown that about 50 per cent of gastric carcinomas contain evidence of a striated border to their cells, and about 30 per cent secrete mucus of intestinal type. When these figures are taken into account an estimate that about 30 per cent of gastric carcinomas arise from areas of intestinal metaplasia appears to be reasonable.

The topographical distribution of intestinal metaplasia and primary carcinoma in the stomach is similar. In stomachs removed for duodenal ulcer, gastric ulcer and carcinoma it is always the pylorus that is revealed as the site most frequently and extensively affected by intestinal metaplasia (Morson, 1955). It is also true that primary carcinoma of the stomach is most frequently found at the pylorus. According to Willis (1953) about half of all gastric carcinomas occur at this site. In the series considered in this study 45 per cent arose from the pyloric part of the stomach, 22 per cent from the lesser curvature, and 18 per cent from the region of the cardia. Only one carcinoma was involving the fundus, and three occupied the region of the greater curve. The remainder (11 out of 107 cases) involved more than half the whole stomach. This order of frequency is similar to the distribution of intestinal metaplasia. It has been shown that the incidence and extent of intestinal metaplasia decreases in the order: pylorus, lesser curve, greater curve and fundus. The similarity in the distribution can be further compared when it is remembered that the gastric canal is the part of the stomach most frequently and extensively affected by intestinal metaplasia (Morson, 1955) and accounts for nearly 70 per cent (excluding the cardia) of all cases of gastric carcinoma. If some cases of carcinoma arise from areas of intestinal metaplasia then one would expect the distribution of these two conditions to be similar.

It has been shown that nearly 80 per cent of a series of 119 stomachs removed for duodenal ulcer, gastric ulcer and carcinoma contain areas of intestinal metaplasia (Morson, 1955). In many of these the gastric mucosa was very extensively replaced by epithelium of intestinal type. Further, in most of the cases of carcinoma in which the transition from intestinal metaplasia to carcinoma can be seen the surrounding mucous membrane showed complete intestinal metaplasia. It would not be surprising from these facts alone if some cases of gastric carcinoma arise from epithelium of intestinal type. It is always easier to demonstrate the type of tissue from which a growth is arising by the study of very early carcinomas. In these the type of mucous membrane adjacent to the tumour gives evidence of its histogenesis. In Example No. 1, the primary carcinoma is a very small one and is only just beginning to invade the stomach wall. The mucous membrane around it (Fig. 3) shows complete metaplasia to an intestinal type of epithelium. In fact, almost the whole of the stomach lining in this case shows

intestinal metaplasia. It is difficult to believe that this tumour arose from ordinary gastric mucosa.

There have been reports in recent years which suggest an inordinately high incidence of gastric carcinoma in patients with pernicious anaemia (Rigler and Kaplan, 1947; Mosbech and Videbaek, 1950). The gastric lesion in pernicious anaemia has been described by Magnus and Ungley (1938) and Magnus (1952). They have shown that it consists of a profound atrophy of all coats of the stomach wall that is localized in its distribution to the body and fundic mucosa. The mucous membrane of the pyloric antrum remains essentially normal. Further, in several of their cases large areas of intestinal metaplasia were found in the atrophic body mucosa, but not in the normal pyloric mucosa. Only one of the 107 cancerous stomachs in this series was removed from a patient with pernicious anaemia. It showed extensive intestinal metaplasia of the body and fundus of the stomach, and a normal pylorus. Schell, Dockerty and Comfort (1954) also report that all their 48 surgical specimens of gastric carcinoma which also had pernicious anaemia showed " hyperplastic islands of intestinalization " at the fundus of the stomach. If a substantial proportion of gastric carcinomas arises from areas of intestinal metaplasia and the distribution of intestinal metaplasia in the stomachs of persons with pernicious anaemia is largely confined to the body and fundic mucosa, then one would expect the majority of primary carcinomas of the stomach in pernicious anaemia to arise from the proximal half of the stomach. Schell, Dockerty and Comfort (1954), in a study of cases in which pernicious anaemia and gastric carcinoma occurred together, have shown that the majority of their tumours arose from the fundus and cardia of the stomach, and not from the pyloric region. This could be explained by the distribution of intestinal metaplasia in pernicious anaemia. However, judgment on this point must be deferred, for Mosbech and Videbaek (1950) state that patients with pernicious anaemia do not develop their gastric carcinomas more frequently at the fundus and body of the stomach, and quote a number of other investigators in support of this.

SUMMARY AND CONCLUSIONS.

1. Five examples of gastric carcinoma have been described which appear to be arising from epithelium of intestinal type.

2. Evidence has been considered which suggests that about 30 per cent of gastric carcinomas arise from areas of intestinal metaplasia in the gastric mucosa.

3. The significance of this in relationship to the increased incidence of gastric carcinoma in patients with pernicious anaemia has been discussed.

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INTRACRANIAL MANIFESTATIONS OF MALIGNANT LYMPHOMA

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ALTHOUGH paraplegia is a well known and not uncommon complication in Hodgkin's disease, intracranial manifestations are comparatively rare in this and other forms of malignant lymphoma.

In the nine-year period ending in 1953, 7 cases have been seen at this hospital in which there has been clinical evidence of the presence of intracranial lymphomatous deposits. In this report we have reviewed the relevant literature and described the clinical and pathological findings in those cases showing intracranial abnormalities among a total of 347 cases of malignant lymphoma, 93 of which were examined at autopsy.

NOMENCLATURE.

The confusion that exists over the nomenclature and classification of these tumours makes it extremely difficult to interpret many of the published reports. The scheme outlined by Robb-Smith (1938) is now generally considered to be too complicated for general use, and even the simpler classification of Gall and Mallory (1942) presents certain difficulties in practice. In this report, we have used the term "malignant lymphoma" to describe a neoplasm of multicentric origin arising in lymphatic tissue. We have adopted four broad histological sub-divisions :

	Gall and Mallory (1942).
Reticulum-cell sarcoma	Stem-celled lymphoma. Clasmatocytic lymphoma.
Lymphosarcoma	Lymphoblastic lymphoma. Lymphocytic lymphoma (including lymphatic leukaemia and mycosis fungoides).
Hodgkin's disease	Hodgkin's lymphoma. Hodgkin's sarcoma.
Giant follicular lymphoma	Giant follicular lymphoma.

MATERIAL REVIEWED.

We have reviewed the records and autopsies of all patients diagnosed as suffering from malignant lymphoma in this hospital between 1946 and 1953. This period has been chosen because most of these cases were seen personally by one of us.

The cases and autopsies were made up as follows :

Histological diagnosis.	Cases.	Autopsies.
Reticulum-cell sarcoma	18	6
Lymphosarcoma	110	41
Hodgkin's disease	125	46
Giant follicular lymphoma	4	0
Unclassified lymphoma	90	0
Totals	347	93

Thirty-nine of the 110 cases classified as lymphosarcoma showed the haematological or bone marrow changes of lymphatic leukaemia. In 4 of the cases of Hodgkin's disease the autopsies were performed at other hospitals; they are included because detailed reports and slides were available for study.

HODGKIN'S DISEASE.

One of the first cases of "lymphadenoma" to be described (Murchison, 1869–70) was found at autopsy to have invasion of the dura mater of the posterior fossa. This patient had not had any clinical evidence of neurological disturbance and there are a few other reports of symptomless intracranial deposits of Hodgkin's disease being found at autopsy (Welch, 1910; Ginsberg, 1927; Rizzi, 1938).

Only 12 cases have been found in the literature of intracranial deposits of Hodgkin's disease giving rise to clinical manifestations. In 8 of these there was confirmation by post-mortem examination. Details of these cases are given in Table I.

TABLE I.—*Hodgkin's Disease with Clinical Evidence of Intracranial Involvement and Autopsy Confirmation.*

Case No.	Authors.	Duration of illness.	Neurological findings.	Pathological findings.
1	von Hecker and Fischer (1922)	8 months	Two days before death, loss of consciousness and epileptic fits	Cherry sized nodule near the centrum ovale. Histology typical of Hodgkin's disease.
2	Sternberg (1925)	1 year	Three months before death, signs of cerebral compression, paralysis of eye muscles and XIIth cranial nerve. Diabetes insipidus	Deposits in parietal, frontal and sphenoid bones. Nodules on inner surface of the dura. Hypophysis replaced by tumour.
3	Colrat (1921)	21 months	A few days before death, blindness, Jacksonian fits involving right side of body and deviation of head to the right	Right side of cerebellum adherent to tumour of dura. Small tumour in dura at occipital pole.
4	Serebrjank (1933)	3½ years	Six months before death, headache, vomiting, ptosis, diplopia and bilateral paralysis of VIth cranial nerve	Small deposits in pons, corpus striatum and white matter of cerebrum.
5	Martin and Courville (1936)	2½ years	In terminal stages, evidence of raised intracranial pressure	Widespread lobulated deposits on innerside of dura, most marked on right side.
6	Winkleman and Moore (1941)	17 months	Presenting symptoms: headache, photophobia, diplopia. Left hemiparesis. Extensor plantar responses	Flat irregular plaques on cerebral surface of the dura.
7	Kohut (1946)	10 months	Eleven months before death, diagnosed as having encephalomyelitis	Diffuse infiltration of dura and leptomeninges.
8	Fein and Newill (1954)	7 years	Seventeen months before death, epileptic fits, hemiplegia, papilloedema	Mass originating in the frontal bone and invading and compressing the frontal lobes.

In our series of 125 cases there was one patient with unequivocal evidence both clinical and pathological, of intracranial deposits of Hodgkin's tissue. Another patient had suggestive symptoms that were improved by treatment with nitrogen mustard, but no autopsy was performed. Two other cases had terminal neurological signs, but no pathological evidence is available of their causation. One patient developed symptoms of brain stem involvement in the course of the disease, but no evidence of intracranial infiltration was found post-mortem. In addition to these cases, intracranial abnormalities were found at autopsy on four occasions but they were of a non-specific type and there was no infiltration with neoplastic tissue. Two patients had large cerebral haemorrhages, the third had atrophy of the frontal lobes and in the fourth the meninges were thickened and small cysts were found in the corpus striatum.

Clinical features.

Intracranial deposits of Hodgkin's disease may be associated with symptoms of raised intracranial pressure, including the development of generalised convulsions or they may cause focal signs suggestive of a space-occupying lesion.

Martin and Courville (1936) described a patient who showed evidence of raised intracranial pressure in the terminal stages of Hodgkin's disease (Table I, Case 5). The case reported by von Hecker and Fischer (1922) developed generalised fits shortly before death and at autopsy an intra-cerebral deposit of Hodgkin's tissue was found (Table I, Case 1). Ginsberg (1927) described two patients shown by biopsy to be suffering from Hodgkin's disease, in whom there were terminal convulsions, but no post-mortem examinations were made. In 5 of the remaining cases in Table I there were focal signs of space-occupying lesions: hemiparesis (Cases 6 and 8), Jacksonian fits (Case 3), cranial nerve palsies (Cases 2 and 4)—in the former complicated by diabetes insipidus. Cases of this type without autopsy confirmation have been reported by Johnsson (1931) and Viets and Hunter (1933). The latter described two patients, one with facial palsy and one with dysphagia, ptosis and VIth cranial nerve palsies. Johnsson's patient had a facial palsy, sensory loss in the tongue and gums and atrophy of the trapezius and deltoid muscles.

Apparently, it is very rare for the intracranial deposits to give rise to the initial symptoms in a case of Hodgkin's disease. Winkleman and Moore's (1941) patient first complained of headache, photophobia and diplopia and only later developed lymphadenopathy, splenomegaly and a Pel-Ebstien fever. Diagnosis was made at this stage by lymph node biopsy. Hemiparesis and cranial nerve palsies developed subsequently. In the other cases the neurological disturbances arose during the course of previously diagnosed Hodgkin's disease and often in its terminal stages.

It is not always safe to attribute neurological disturbances developing in advanced cases of Hodgkin's disease to intracranial deposits of lymphoma. Bateman, Squires and Thannhauser (1945) described a patient diagnosed as having Hodgkin's disease from lymph gland biopsy, who two years later developed blindness, mental deterioration and increasing weakness associated with dysarthria and IIIrd nerve palsies. At autopsy evidence of Hodgkin's disease was found in the lymph glands, liver and spleen, but none inside the cranium. The brain was atrophied, softened and showed cystic changes in the choroid plexus. The clinical course was considered to be too rapid for a neoplastic process

and the pathological changes were not those of radiation damage. The authors of the report thought that the findings may have been due to secondary toxic changes from the Hodgkin's deposits elsewhere. Adams, Denny-Brown and Foley (1953) described a similar case in which they thought that the absence of a lesion at autopsy was the result of irradiation. Gray, Baker, Cotrell and Kogland (1941) attribute neurological symptoms in the absence of demonstrable infiltration of the C.N.S. at autopsy, to pressure of enlarged glands in the neck and thorax on the great veins and the veins of the vertebral group leading to cerebral oedema. Hoster, Dratman, Craver and Rolnick (1948) suggest that a toxic encephalitis arising in the course of the disease may be the cause of intracranial or spinal signs. Neurological manifestations pointing to involvement of the spinal cord have also been described in patients in whom no abnormality has been found at post-mortem to explain them (Gray *et al.*, 1941). Whatever the explanation of this intriguing phenomenon it makes it difficult to accept as authentic, cases in which intracranial deposits of Hodgkin's tissue are thought to be present on clinical grounds alone. Post-mortem or other objective evidence appears to be essential.

Primary Hodgkin's sarcoma of the brain

Three cases of this condition have been described by Kinney and Adams (1943) and by Sparling, Adams and Parker (1947). All three presented with neurological symptoms, the first with drowsiness, confusion and speech difficulties; the second with ataxia, confusion, incontinence and dysphagia; and the third with ataxia, diplopia and hemiplegia. Clinical examination of all three patients suggested the presence of an intracranial space-occupying lesion, but in none of them was there any abnormality of the lymphatic glands, liver or spleen. In the first case a tumour of the left frontal lobe was removed at operation but the patient died; a complete autopsy was carried out and no other deposits of tumour were found. The other two cases died; post-mortem examination was limited to the head and in each one a cerebellar tumour was found. All three of these tumours showed histological appearances consistent with Hodgkin's sarcoma. In the absence of any evidence of a multicentric origin we agree with Troland, Sahyoun and Mandeville (1950) that these tumours should not be included among the malignant lymphomas. These authors suggest that reticulum-cell sarcoma and Hodgkin's sarcoma arising primarily in the brain should be grouped together with perithelial and spindle-celled sarcomas of the brain as " primary mesenchymal tumours of the brain ".

Case Reports:

CASE 1.—The patient was a very intelligent young man; when first seen at this hospital in 1948 he was 21 years of age. He had noticed a lump in his neck in 1947 and biopsy early in 1948 showed the changes of Hodgkin's disease. Deep X-ray therapy was given and the glands rapidly disappeared. The patient remained well until mid-1949 when he started to get pain in the back radiating down the legs. There were no deposits visible on X-ray examination of the lumbar spine, but irradiation of this area eased the pain. During 1951 there was evidence that the disease was becoming more generalised; the patient developed breathlessness, malaise and night sweats. A course of nitrogen mustard was given with

considerable symptomatic relief and later in the year a second course was given because pain in the back had become very troublesome. The injections diminished this pain.

In February, 1952, he complained of severe headaches and mistiness of vision. On examination there were retinal haemorrhages and gross papilloedema, but no other abnormal signs could be found in the central nervous system. Both blood pressure and renal function were within normal limits. The headaches and vision were improved by a further course of nitrogen mustard, but during the next three months he had several epileptic fits. In June, 1952, the patient reported that he had lost his sense of musical pitch and had noticed a disturbance of position sense in his arms. The headaches had once again become very severe and his eyesight had further deteriorated. Examination showed an increase of the papilloedema and on this occasion it appeared a little more marked on the right side. The only other abnormality in the central nervous system was an increased left ankle jerk.

He was transferred to the National Hospital for Nervous Diseases under the care of Dr. William Gooddy and Mr. Wylie McKissock, to whom we are indebted for permission to quote the following information :

On admission there, his physical signs were unaltered. X-ray of the skull showed no abnormality, but an E.E.G. was interpreted as suggestive of a midline or posterior fossa lesion. Ventriculography showed that the septum lucidum was displaced to the left of the midline, the right temporal horn was slightly elevated, indicating a large space occupying lesion in the right occipital area. The ventricular C.S.F. was faintly turbid with 2 cells/cmm. and 45 mg. of protein/100 ml.

Craniotomy was performed by Mr. L. S. Walshe. A right tempero-parietal flap was raised and a quadrilateral bone flap based on the temporal muscle was cut and elevated. The dura in the inferior part of the exposure was discoloured and adherent to a yellowish grey mass. An attempt was made to separate this from the adjacent brain, which appeared to be infiltrated. The mass extended inferiorly along the floor of the middle fossa and as much as possible was excised, but it was noted that tumour tissue still extended medially.

A report on the tissue removed was made by Dr. J. G. Greenfield. It was a mass weighing 28 g., well vascularised and composed of connective tissue with numerous small islets of cellular deposits. The cells were mainly rounded or elongated reticulum cells with similar shaped nuclei. Cells in mitosis and cells with hyperchromatic nuclei were also present. There were many eosinophils and giant cells with a single large or multi-lobulated nucleus. The appearances were suggestive of Hodgkin's disease (Fig. 1).

Postoperatively, the patient described attacks of sensations of floating on air and he suffered uncontrolled outbursts of emotion. Examination at this time showed a left anosmia, there was no papilloedema although the retinal vessels were overfilled. The right knee and ankle jerks were absent. The patient was transferred back to this hospital and his generalised disease was treated with triethylene melamine (T.E.M.) and radiotherapy. In early 1953 he had an attack of hiccoughing which was thought to be central in origin as it subsided after two days deep X-ray therapy to the region of the medulla.

During the summer of 1953 he suffered considerable pain in his back and abdomen and had a further episode of hiccoughing, but at no time before his

death in September, 1953, did abnormal signs appear in the central nervous system.

At autopsy there were bilateral pleural effusions but no pulmonary deposits. The liver was normal in size, but had white nodules of Hodgkin's disease scattered through it. The spleen was much enlarged and had appearances typical of Hodgkin's disease. Lymph glands in the abdomen and thorax were enlarged and white, and the dorsal vertebrae contained irregular areas of sclerosis and discoloration. Under the craniotomy flap the brain was adherent to the meninges. At this site there was a mass of firm white tissue looking like the abnormal lymph glands. There was marked softening of the underlying brain.

Histological examination of this dural mass showed it to be a fibrous deposit of Hodgkin's disease with many giant cells scattered throughout the section. There was no evidence of infiltration of the surrounding brain (Fig. 2).

Comment.—This patient had a slowly progressing variety of Hodgkin's disease. Some years after the disease first became clinically obvious he developed evidence of a raised intracranial tension. The headache and papilloedema were only temporarily improved by chemotherapy, and although the patient's general condition remained quite good it seemed likely that blindness would soon result from the optic nerve changes. Investigation and operation were undertaken with removal of a considerable portion of the tumour and relief of his symptoms. Subsequent chemotherapy and irradiation may have helped to control any recurrence. The patient lived for fourteen months, able to read and remaining in full possession of his faculties although gradually becoming weaker as the disease progressed.

CASE 2.—The patient was a married man aged 38 who, towards the end of 1951, noted some lumps in the neck. Biopsy at the Luton and Dunstable Hospital in February, 1952, showed typical appearances of Hodgkin's disease. After the operation he had no symptoms or clinical evidence of other deposits of the disease and no treatment was advised. In September, 1952, he developed an acute illness with bi-temporal headaches, sweating, vomiting and sleeplessness. After five days he was admitted to the Luton and Dunstable Hospital under the care of Dr. T. Parkinson. On examination he was feverish and had neck rigidity, the palate moved to the right on phonation and the tongue was protruded to the right. There were no other abnormal signs in the central nervous system. Lumbar puncture gave a C.S.F. pressure of 200 mm. and the fluid contained 11 lymphocytes/cmm. and 20 mg. of protein/100 ml. The W.R. was negative. After two weeks a left facial palsy developed. It was suggested that he had a brain-stem lesion with meningeal infiltration. After seven weeks in hospital, the facial weakness had cleared although he still had some headache. On discharge the C.S.F. contained 16 lymphocytes/cmm. and 55 mg. of protein/100 ml.

He never felt well after this illness and in February, 1953, he was sent to University College Hospital for deep X-ray therapy. The response to a course of treatment was poor and he was admitted for chemotherapy with T.E.M. On admission he had generalised lymphadenopathy and hepato-splenomegaly, but no abnormal signs were found in the central nervous system. The initial response to chemotherapy was good, but there was a rapid relapse, and in September, 1953, he died two weeks after a profuse epistaxis.

At autopsy, there were large hilar, tracheo-bronchial and mediastinal glands. The liver and spleen showed extensive tumour deposits. The contents of the skull were normal except for two shallow pigmented ulcers on the surface of the left temporal lobe. Histological examination of the glands, liver and spleen showed changes typical of Hodgkin's disease. In the brain there was gliosis and iron pigmentation in the region of the ulcers suggestive of an old haemorrhage. There was no evidence of lymphomatous infiltration.

Comment.—This patient, during the course of a rather acute Hodgkin's disease, had an illness in which neurological signs and changes in the C.S.F. suggested a brain-stem lesion. The neurological signs cleared without treatment and no evidence of intracranial Hodgkin's disease was found at autopsy. No explanation can be given for the neurological disturbance. It may have been unrelated to the Hodgkin's disease or it may have represented an example of the " toxic encephalitis " described by Hoster *et al.* (1948).

CASE 3.—A young man aged 25 was discharged from the Army in 1942 with a diagnosis of Hodgkin's disease. In 1943 he was seen at the Shaftesbury Hospital with enlarged glands in the neck, right axilla and groins. Chest X-ray showed enlarged mediastinal glands and further biopsy confirmed the diagnosis. He was referred to the Radiotherapy Department at U.C.H. and given a course of deep X-ray therapy, to which he responded moderately well. In 1945 he was admitted for further treatment and complained of numbness of the anterior abdominal wall for which no obvious cause could be found. By 1948 he had lost a lot of weight and complained of vague abdominal pain. The spleen, liver and lymph glands were much enlarged and he was admitted for a transfusion and further deep X-ray therapy.

In September, 1948, he was re-admitted because of three or four attacks in the preceding three weeks. These attacks took the form of trembling of the lips and hands associated with a sensation of impending death, and during two of these attacks he lost consciousness. He complained of headaches when reading and attacks of morning vomiting. On examination, the only abnormal finding in the central nervous system was early bilateral papilloedema. During this admission he had one further attack and on this occasion did not lose consciousness. The attacks stopped after a course of nitrogen mustard therapy and he was discharged home in October, 1948. He deteriorated rapidly and was re-admitted to the Shaftesbury Hospital, where he died in December, 1948. No post-mortem examination was carried out.

Comment.—In the absence of autopsy confirmation this case can only be put forward as a presumptive case of intracranial Hodgkin's disease. Late in the course of a slowly progressive form of disease focal epilepsy developed and papilloedema was observed. The symptoms were relieved by nitrogen mustard therapy although the patient died of weakness and anaemia three months later.

CASE 4.—A single woman developed Hodgkin's disease when she was 38 years old. The presenting features were enlarged glands in the neck and they responded well to irradiation. Later in the year she developed lesions in the pelvis and femur, for which further treatment was given. Over the next four years her disease ran a slowly progressing course, further irradiation and chemotherapy producing only slight remissions. She was greatly troubled by generalised

pruritus and in the terminal stages by pain from a pathological fracture of the neck of the femur. One month before death the pain and irritation ceased to trouble her, she had a series of Jacksonian epileptic attacks starting in the face and there were signs of an upper motor neurone lesion affecting the left side of the body. It was concluded that she had a lesion involving the frontal lobe and motor area, but she died at home and no autopsy was performed.

CASE 5.—A schoolgirl aged 14 developed pain in the chest and recurrent fever. Mediastinal glands were shown to be enlarged on X-ray, and biopsy of a neck gland confirmed that the patient had Hodgkin's disease. Deep X-ray treatment was given with good response. During the next four years treatment was given to various groups of glands and also to a number of bone deposits that gave rise to pain and were demonstrated radiologically. Six weeks before she died she developed mental changes and had a series of Jacksonian fits affecting the left side of the body. No autopsy was performed.

Comment.—Cases 4 and 5 were both examples of slowly progressing Hodgkin's disease. In the terminal stages epileptic fits and signs suggesting a focal cerebral lesion were observed. Unfortunately neither of these cases were examined post-mortem.

LYMPHOSARCOMA.

Under the heading of lymphosarcoma we have included conditions often described as lymphoblastic or lymphocytic lymphoma, mycosis fungoides and acute and chronic lymphatic leukaemia. In all these diseases the histology of the individual lesion is the same, there being a dense accumulation of rather uniform lymphoblasts or lymphocytes. In the lymphatic leukaemias there is widespread infiltration of the bone marrow with a spill-over of the cells concerned into the peripheral blood. The earlier literature about conditions falling into this group is extremely confused and it is very difficult to decide which cases should be included.

Review of the relevant literature has failed to disclose any case of mycosis fungoides with evidence of intracranial involvement. A few cases of lymphosarcoma with this complication have been recorded, and there have also been descriptions of series of cases of either the leukaemias as a whole or of lymphatic leukaemia in which evidence of intracranial involvement has been claimed in a surprisingly high percentage (20 per cent). The cases reported in the literature with intracranial involvement by lymphosarcoma, without leukaemic changes in the blood, are shown in Table II. Only cases in which histological diagnosis appeared to be definite and in which there was autopsy confirmation have been included. In most of the cases cranial nerve palsies were an early and striking feature. Davidson and Michaels (1930) (Table II, Case 1) described the case of a 45-year-old man who, one and a half years after the onset of cervical lymphadenopathy, developed IIIrd and VIIth nerve palsies, these improved with radiotherapy but were followed by evidence of a hemiplegia. At post-mortem examination the lesions were found to be primarily in bone and dura, with compression of the cerebrum and the brain stem. These authors also described two cases with less striking cranial nerve involvement, but in neither was autopsy performed. Young, Young and Gysin (1945) (Table II, Case 4) reported the case of a youth aged 17 who developed headache followed by a right facial weakness and who was later

TABLE II.—*Cases of Generalised Lymphosarcoma—without leukaemia—with Clinical Evidence of Intracranial Deposits and Autopsy Confirmation.*

Case No.	Authors.	Duration of illness.	Clinical findings.	Pathological findings.
1	Davidson and Michaels (1930)	2 years	Five months before death; cranial nerve palsies, papilloedema and hemiparesis	Mass originating in skull bones, compressing pons and medulla.
2	Viets and Hunter (1933)	10 months	Three months before death; dysarthria, Jacksonian fits, visual field defects	Widespread cerebral deposits.
3	Radzinski and Uznanski (1944)	2 weeks	Presented with meningism, papilloedema, diplopia, nystagmus, facial nerve weakness. Fits in terminal stage	Brain macroscopically normal, dense meningeal and perivascular lymphomatous infiltration in sections.
4	Young, Young and Gysin (1945)	3 months	Presented with signs of intracranial tension, cranial nerve palsies, and generalised muscle weakness	Infiltration of meninges and cranial nerves destroyed.
5	Halpern (1952)	5 weeks	Presented with facial paralysis and pain in the leg	Disseminated foci and invasion of cranial nerve nucleii.

found to have papilloedema, ptosis and bilateral VIIth nerve palsies. At autopsy there was infiltration of the IIIrd and VIIth nerve sheaths with more widespread involvement of the meninges and perivascular spaces. Halpern (1952) (Table II, Case 5) also reported a case of lymphosarcomatosis in which there were bilateral VIIth nerve palsies due, in this case, to focal infiltration of the nuclei of the cranial nerves concerned. The case reported by Radzinski and Uznanski (1944) (Table II, Case 3) presented with symptoms and signs suggesting meningeal involvement in addition to partial VIIth nerve weakness on both sides. Post-mortem examination revealed a very dense lymphocytic infiltration of the meninges, especially of the perivascular spaces.

A case reported by Viets and Hunter (1933) (Table II, Case 2) probably falls into the lymphosarcoma group and this patient presented somewhat differently. She developed fullness on the nasal side of one eye followed by Jacksonian epilepsy and evidence of unilateral cerebral involvement. At autopsy there were widespread deposits of lymphosarcoma with extensive infiltration of the right cerebral hemisphere. Abbott and Adson (1943) report four additional cases of malignant lymphoma involving the central nervous system. In two it is difficult to be certain about the exact histological diagnosis, but the principal clinical features were cranial nerve palsies. Both these patients had slowly-growing neoplasms and there was some improvement following radiotherapy. Autopsies were not performed. The other two cases showed leukaemic changes in the peripheral blood and are discussed in the next section.

In three of the cases shown in Table II neurological symptoms were the presenting feature. In the two of these (Cases 3 and 5) clinical evidence of deposits in other organs indicated the widespread nature of the disease process, but in the other

(Case 4) there was little indication as to the origin of the neurological disturbance. Lumbar puncture in this case showed a pressure of 550 mm. and the C.S.F. contained 1300 cells/cmm., of which 95 per cent were mononuclear. Case 3 had increased pressure on lumbar puncture with 2144 cells/cmm., 98 per cent of which were lymphocytes. In the cases described with intracranial lesions the disease ran a rapid course and the histological descriptions suggest that the predominant cell type was very immature (they would be regarded as lymphoblastic rather than lymphocytic lymphomas). The nature of the pathological lesions should be noted: meningeal infiltration in two, focal infiltration of the brain in two and lesions originating in the bones and meninges in one. In no case was there any evidence of intracranial haemorrhage.

In contrast to these findings is the reported high incidence of intracranial disturbance in cases of leukaemia. In many of the reported series myeloid and lymphatic leukaemia are considered together. In the cases described by Diamond (1934) there were four patients with lymphatic leukaemia in whom evidence of intracranial involvement was found post-mortem. Only one patient had any clinical evidence of this complication, and he presented with headache and IIIrd and VIIth cranial nerve weakness. At autopsy there was microscopic evidence of infiltration of the cerebellum and brain-stem as well as some perivascular cuffing. Two of the patients described by Veits and Hunter (1933) had a malignant lymphoma associated with lymphatic leukaemia. One of these had terminal pyramidal signs associated with intracerebral and meningeal foci of lymphomatous infiltration. In the second patient there was clinical evidence of cranial nerve and cerebral disturbance, but no autopsy was performed. Schwab and Weiss (1935) reported a case of a young man with acute lymphatic leukaemia who complained of dysphagia, loss of taste and facial weakness. These authors made repeated observations of the changes in the cerebro-spinal fluid during this patient's illness of 139 days. On the third day the C.S.F. pressure was 150 mm. and there were 1900 lymphocytes/cmm. with a protein of 170 mg./100 ml. On the 60th day, the pressure was 210 mm. with 4500 lymphocytes/cmm. and a protein of 426 mg./100 ml. Subsequently the pressure became normal and the cells fell to 25–50 lymphocytes/cmm. with a protein of 125 mg./100 ml. At autopsy, focal cerebral deposits and diffuse meningeal infiltration were found.

Schwab and Weiss (1935) reviewed 334 cases of leukaemia and reported clinical evidence of involvement of the central nervous system in 17 patients out of 80 with chronic and 14 out of 62 with acute lymphatic leukaemia. Except for the case described above, details of the clinical features on which these figures are based are not reported. It is not clear how often the disturbance was due to intracranial changes, and autopsy confirmation was unusual, only 3·3 per cent of their cases came to post-mortem examination. They also collected data from the literature up to 1935 of 29 cases of acute and 17 cases of chronic lymphatic leukaema in which there was pathological evidence of involvement of the central nervous system. Leidler and Russell (1945) reported 5 cases of lymphatic leukaemia involving the brain, in none of these cases was there any clinical evidence of intracranial involvement. The type of pathological lesion which they described was usually a microscopic haemorrhage with surrounding aggregation of lymphocytes, perivascular cuffing or scatttered cells in the subarachnoid space. In only one case was there a definite nodular leukaemic infiltration. They also survey the cases of all types of leukaemia in the literature up to 1945 in which pathological

evidence of intracranial involvement was found at autopsy. They analysed 67 cases, including 20 of their own. Of these, 11 showed evidence of a hemiplegia during life and all died of cerebral haemorrhage. Of the others 36 were found at autopsy to have some form of haemorrhage into the brain. Unfortunately, platelet counts or comments on clinical evidence of a haemorrhagic tendency are seldom given in these reports. Sparling, Adams and Parker (1947) claim that 20 per cent of cases of lymphatic leukaemia are found to have evidence of neurological involvement at autopsy.

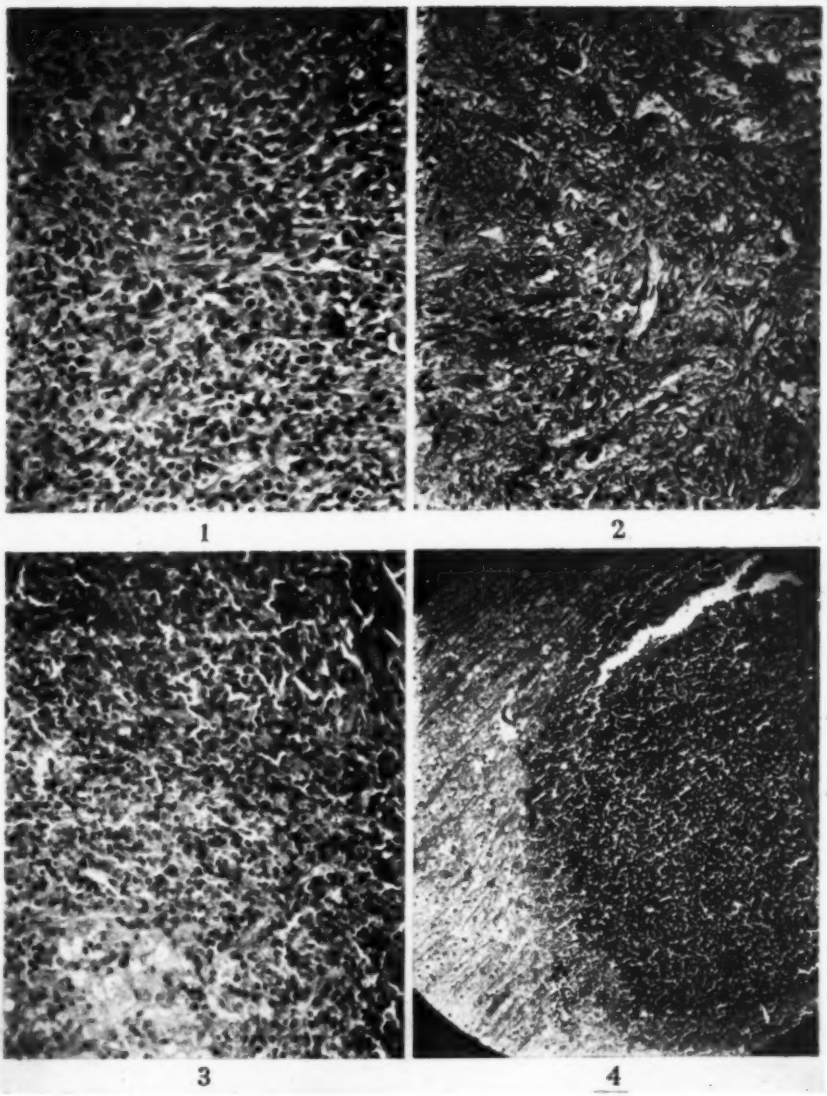
Critical examination of the reported data suggests that cases of lymphatic leukaemia showing clinical evidence of intracranial involvement which has been confirmed at autopsy are comparatively rare. The clinical manifestations are not unlike those found in cases of lymphosarcoma without leukaemic changes. Cranial nerve palsies were described in two cases, in one associated with meningeal infiltration and in the other, with focal leukaemic deposits. It is questionable whether the pathological changes that are reported in cases of leukaemia are always to be regarded as leukaemic infiltrations. The haemorrhages with surrounding lymphocytes so often described are probably associated with a low platelet count, and in patients with very high lymphocyte counts in the peripheral blood minute collections of cells in the subarachnoid and perivascular spaces are not necessarily evidence of leukaemic infiltration.

Two cases were described by Abbott and Adson in 1943 under the heading of " Primary Intracranial Lymphosarcoma ". In one case the tumour originated in the dura and in the second in the skull bones. In the first of these cases, at autopsy, there was no evidence of lymphoma elsewhere, and this case should probably be grouped with the primary mesenchymal tumours of the brain rather than as a malignant lymphoma. In the second case, the presenting features were ataxia and a lump in the fronto-pariental region. Craniotomy was thought to show chronic inflammatory tissue only. Subsequently, however, glands appeared in the neck and biopsy of one of these was said to show a lymphosarcoma. The patient died seven years after the onset of the symptoms, but no post-mortem examination was carried out. Review of the histology suggested that the tumour arose in the bone, but its exact nature remains uncertain. There is nothing in the description given to suggest the widespread dissemination that would have been expected with a malignant lymphoma of seven years duration.

In our series of cases of malignant lymphomas we have observed 110 patients with tumours that we have grouped as lymphosarcoma. Of these, 39 had leukaemic changes in the peripheral blood. There was clinical evidence of involvement of the intracranial contents in one patient with lymphosarcoma without leukaemia and one patient with acute lymphatic leukaemia died of a cerebral haemorrhage. Post-mortem confirmation of the intracranial involvement was found in the first patient; in the second although there was no macroscopic evidence of infiltration, definite nodules of neoplastic tissue were found on microscopy.

EXPLANATION OF PLATE.

FIG. 1.—Case 1: Section of intracranial deposit removed at operation.
FIG. 2.—Case 1: Section of intracranial deposit at autopsy.
FIG. 3.—Case 6: Section of intracranial mass.
FIG. 4.—Case 7: Section showing one of the intracerebral deposits.



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3 4

John and Nabarro.

We cannot give any figures for the incidence of microscopic evidence of involvement of the brain or meninges in these cases because extensive histological study of these tissues was not undertaken.

Case Reports.

CASE 6.—In 1947, at the age of 46, the patient noted enlarged glands in his neck. Biopsy of one of these was reported as showing a malignant lymphoma. The glands responded to deep X-ray therapy given early in 1948, but shortly afterwards he developed pain behind the right eye, prominence of the right eye and double vision. He also complained of a purulent nasal discharge. X-rays showed infiltration of the frontal, ethmoidal and maxillary sinuses with partial destruction of the roof of the orbit, In June, 1948, he developed girdle pain and a complete paraplegia below the level of the 10th dorsal segment. On admission, he was found to have a right-sided proptosis, paralysis of the muscles of the right eye, movement of the palate to the right on phonation and signs of paraplegia. In addition, there were enlarged glands in the neck, axillae and groins, and a number of skin nodules. Following a course of nitrogen mustard injections the proptosis and glands regressed and the nasal discharge ceased, but there was no change in the paraplegia. His general condition continued to deteriorate rapidly and he died ten days after the completion of the course of nitrogen mustard.

At autopsy, the glands, including those of the mediastinum, were enlarged and infiltrated with yellow lymphoma. The brain was soft and oedematous. Anteriorly, apparently originating in the orbit, was a mass of greenish yellow necrotic tissue infiltrating the base of both frontal lobes. The tumour extended back to the optic chiasma, but the pituitary stalk was free. Both ventricles appeared normal and there was no evidence of raised intracranial pressure. Microscopically the tumour was extremely necrotic in places, but elsewhere the appearances were suggestive of a lymphoblastic lymphoma (Fig. 3).

Comment. In this case there was a lymphoblastic lymphoma originating in the bones or tissue in the roof of the orbit and actually invading the brain at its base. The principal clinical evidence of intracranial involvement was a unilateral palatal weakness.

CASE 7.—The patient was a woman of 67, admitted to hospital with a history of general weakness, breathlessness and pallor for the preceding two months. On examination she had numerous petechiae, enlarged lymphatic glands, tonsils, liver and spleen. The blood count suggested a subacute lymphatic leukaemia and she improved little with blood transfussion and corticotrophin. Two weeks after admission, she became comatose with signs of cerebral haemorrhage and died within twenty-four hours.

At post-mortem, numerous petechiae were noted and infiltration of the lymph nodes, liver, spleen and bone marrow. There was a large left-sided cerebral haemorrhage with many small haemorrhages in the surrounding brain. Microscopically the haemorrhages were found to be associated with small leukaemic deposits (Fig. 4).

Comment.—This patient developed a cerebral haemorrhage as part of the haemorrhagic tendency of an acute or subacute leukaemia. It is presumably to be related to a thrombocytopenia although no platelet count was done. The nodular leukaemic infiltrations did not produce any clinical abnormality, although

it is possible that they may have played some part in the development of the terminal haemorrhage by damage to the walls of the blood vessels.

RETICULUM-CELL SARCOMA.

Primitive lymphomas in the so-called reticulum-cell sarcoma group present certain difficulties in recognition and in differentiation from other primitive mesenchymal tumours. We have restricted the term reticulum-cell sarcoma to multicentric tumours and have been only able to trace one possible case of this type with involvement of the intracranial contents. This is the second case reported by Abbott and Adson (1943).*

A number of writers have reported cases of primary reticulum-cell sarcoma of the brain. These cases have been reviewed by Troland, Sahyoun and Mandeville in 1950, who conclude that they should be grouped among the primary mesenchymal tumours of the brain and not as malignant lymphomata. In this group they also include cases of primary Hodgkin's sarcoma and primary lymphosarcoma of the brain.

UNCLASSIFIED LYMPHOMA.

In addition to the cases already described, one of our patients with malignant lymphoma of controversial histological type had evidence, clinically, of invasion of the base of the skull and involvement of the cranial nerves. Unfortunately, the patient died at home and no autopsy was performed, so we are unable to give details of the anatomical or histological nature of the neoplasm.

DISCUSSION.

In this report we have been concerned primarily with the clinical manifestations of intracranial malignant lymphoma. Our own cases have confirmed the general impression gained from the literature, that pathological evidence of such involvement is nearly as rare as clinical evidence although we cannot exclude the presence of microscopic deposits in all cases. The number of cases of Hodgkin's disease with intracranial deposits giving rise to symptoms and confirmed by post-mortem found in the literature is eight, and we are able to add one further case. Surgical or autopsy confirmation of such deposits is essential because of the evidence reported by Bateman, Squires and Thanhauser (1945) and Jackson and Parker (1945) of the occurrence of neurological symptoms in these patients without there being any corresponding neoplastic changes found at autopsy. We have reported one further case of this type (Case 2).

In most of the cases of intracranial Hodgkin's disease described in the literature the disease ran a rather rapid course and the neurological symptoms were a late development. Specific treatment has rarely been attempted although Fein and Newill's (1954) patient had irradiation and nitrogen mustard. In our patient there was some relief of symptoms with chemotherapy, but in view of the continued progress of the lesion and the patient's good general condition, surgery was undertaken. The results of this exploration were thought to have justified

* After this paper was submitted Allison and Gordon (1955, *Lancet*, ii, p. 120) reported a case of a reticulum cell sarcoma presenting with neurological symptoms. A cerebral deposit and meningeal infiltration were found at autopsy.

the course; the patient lived a further fourteen months and the intracranial lesion gave him little further trouble. In cases of slowly progressing Hodgkin's disease, in which there is evidence of an intracranial deposit, the possibility of diagnostic studies for the localisation of such a lesion and its operative removal should be considered if the neurological symptoms develop before the disease enters its terminal stage. The types of disturbance that should suggest intracranial deposits are: evidence of raised intracranial tension, Jacksonian fits, cranial nerve palsies or other focal neurological signs.

In lymphosarcoma without leukaemic blood changes evidence of intracranial involvement is equally rare. We have collected five cases from the literature and added one of our own. In these cases the disease has nearly always been a rapidly-growing lymphoma in which the long-term response to treatment has been very disappointing. Four of the patients had cranial nerve palsies due either to nuclear involvement or infiltration of the nerve sheath. In one patient with Jacksonian epilepsy irradiation was claimed to have led to improvement, but the disease was widespread and rapidly proved fatal.

In patients with leukaemic blood changes in association with lymphosarcomatous infiltration of the lymphatic glands and bone marrow the reported incidence of neurological involvement is suprisingly high. The incidence of intracranial involvement is lower, and in many of these the pathological lesion is a cerebral haemorrhage. Whether this is due entirely to the low platelet count or whether some of the haemorrhages result from tumour nodules invading the vessel wall is uncertain. In our series of thirty-nine cases of lymphosarcoma with leukaemic changes in the peripheral blood, there was one patient who died of a cerebral haemorrhage.

Clinical evidence of intracranial involvement in generalised reticulum-cell sarcoma is apparently extremely rare and we have only been able to trace one possible case.* The condition of primary reticulum-cell sarcoma of the brain should probably not be included under the heading of malignant lymphoma.

SUMMARY.

The literature relating to the clinical evidence of intracranial involvement in cases of malignant lymphoma has been reviewed.

Experience of this complication in a series of 347 cases of malignant lymphoma admitted to University College Hospital between 1946 and 1953 is reported. Ninety-three of the patients were examined at autopsy.

From the review of the literature and the findings in this series intracranial deposits of malignant lymphoma appear to be very uncommon.

One case of Hodgkin's disease with an intracranial deposit is reported. Partial removal was undertaken with considerable benefit to the patient. Other cases of Hodgkin's disease included one with symptoms suggesting intracranial deposits although none were found at post-mortem examination, and three with clinical evidence of intracranial manifestations who were not examined at autopsy. In one of these the symptoms were relieved by chemotherapy.

One case of lymphosarcoma invading the base of the brain from the bones of the skull is described. The high incidence of intracranial involvement reported in lymphatic leukaemia has not been confirmed in this series.

* See footnote to p. 398.

We wish to thank Dr. Gwen. Hilton, Director of the Radiotherapy Department at University College Hospital, to whom many of the patients were originally sent. We also would like to thank Dr. J. F. Stokes for permission to include Case 7, Professor G. R. Cameron for allowing us to refer to the autopsy reports, and Mr. E. Bligh for the photographs.

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RETICULUM CELL SARCOMA OF THE THYROID GLAND.

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PRIOR to the 20th century, sarcoma of the thyroid gland was reported almost as frequently as carcinoma (Kocher, 1883; Bégin, 1849; Halsted, 1924) but recent investigations based on a large series of cases have altered the reported incidence. Ewing (1940), convinced that primary thyroid sarcoma did not exist, contended that tumours of the thyroid which had been described as sarcoma were probably anaplastic carcinoma. His positive opinions exerted a great influence on pathologists, especially in the United States where the diagnosis of thyroid sarcoma is made, still, with some hesitancy. Ewing's views, however, are not shared by all investigators, and the influence of Wegelin (1926) and others is reflected in the comparatively frequent reports of thyroid sarcoma in Europe.

Many of the thyroid sarcomata reported in the United States during the past twenty years fall into the lymphoma group. Though agreement in the classification is not complete, lymphomata are frequently separated into three categories, Hodgkin's disease, lymphosarcoma, and reticulum cell sarcoma (Willis, 1948; Richter, 1953; Gall, 1943). This classification will serve the purpose of this report. Lymphosarcoma is the most frequently reported (Rice, 1932; Dinsmore, Dempsey and Hazard, 1949) while Hodgkin's disease and reticulum cell sarcoma are relatively rare. Lymphosarcoma and reticulum cell saroma are usually indistinguishable clinically but the two diseases are sometimes differentiated histologically, and in this report only cases of reticulum cell sarcoma of the thyroid are discussed.

In the previously reported cases, involvement of the thyroid gland was the predominating feature of the disease, but in none of these cases was there incontestable evidence that the reticulum cell sarcoma was primary in the thyroid. The opportunity to prove that this disease may occur as a solitary lesion in the thyroid gland was presented by the post-operative death of a patient having histologically proved reticulum cell sarcoma of the thyroid gland.

Case I.

An 86-year-old white woman was admitted to Garfield Memorial Hospital complaining of a swelling in the left side of her neck which had been present for one month and was associated with pain on swallowing. On physical examination a poorly defined mass was palpated in the region of the left lobe of the thyroid gland, extending slightly over the midline. The cervical, supraclavicular, axillary and inguinal lymph nodes were not enlarged, and no other physical abnormality was found. The vocal cords moved normally.

At operation, the entire left lobe of the thyroid gland was found to be composed of a diffuse tumour. No enlarged cervical nodes were present. A total thyroidectomy was preformed without difficulty, and, as neither vocal cord moved on direct laryngoscopic examination, a tracheotomy was performed.

The gross specimen weighed 68 g. and consisted of two unequal lobes connected by a small isthmus. The left lobe measured 7 × 6 × 5 cm. and the right 5 × 3 × 2 cm. The left lobe was uniformly enlarged and smooth. Cut section showed a smooth, firm, homogeneous, grayish-tan surface, except for a medial rim of brownish-red, mucinous, normal appearing thyroid tissue. The right lobe was normal.

Microscopically (Fig. 1) the tumour consisted of large sheets of closely packed, uniform cells supported by a minimal amount of stroma. The tumour had replaced nearly all of the thyroid tissue in the left lobe, leaving occasional isolated colloid-containing follicles, some of which were undergoing degeneration.

The individual cells were approximately twice the size of lymphocytes and their nuclei were correspondingly large. A moderate amount of homogeneous acidophilic cytoplasm surrounded the nuclei and the cells were outlined clearly. The nuclei were large and unformly round or ovoid with scattered indented forms, and the chromatin was scattered diffusely. In many of the cells a prominent nucleolus was present. A few cells were undergoing mitosis and rare multinucleated giant cells were seen. Wilder stains demonstrated the delicate reticulum intimately associated with individual cells and separating them singly or into groups of two or three.

The right lobe and the isthmus consisted of normal thyroid tissue with follicles of variable sizes, lined by flattened cuboidal cells. Only a few small foci of lymphocytes were present in this lobe.

The patient developed pneumonia after the operation and in spite of intense therapy she died on the fourth post-operative day.

An autopsy was performed three hours after death. The skin incision in the neck was partly healed. A large amount of blood, pus and débris was present, covering the trachea and extending into the tracheotomy opening and down into the bronchi. The anterior neck muscles were hyperaemic and oedematous. There were numerous soft, dark red cervical lymph nodes measuring up to 2 cm. Other lymph nodes in the mediastinal, para-aortic, iliac, mesenteric, axillary and inguinal areas were small and firm. The spleen weighed 110 g., and the liver weighed 1430 g. Both lungs contained patchy areas of broncho-pneumonia.

On microscopic examination, the cervical lymph nodes were hyperplastic and contained a large amount of blood pigment, but there was no evidence of reticulum cell sarcoma. The lymph nodes from the other areas of the body showed no disease or alteration from the normal, and no other tissues contained any evidence of reticulum cell sarcoma. The gross diagnosis of bronchopneumonia was confirmed.

This case was submitted to the Committee on Thyroid Cancer of the American Goiter Association. There was almost unanimous agreement to a diagnosis of primary reticulum cell sarcoma of the thryiod gland.

Case II.

A 74 year-old white woman came to New End Hospital in February 1954, complaining of a swelling of the neck which had been present for several months. A rapid increase in size had occurred in the preceding two months. There was a

history of a goitre fifteen years earlier which had been "cured with medicine". She had recently lost her appetite, her voice had become hoarse, and she had been unduly tired and had suffered from dyspnoea, insomnia, anxiety and depression. The lump in the throat was not painful but she had pain behind her ears and a sensation of constriction, especially when she swallowed. On examination, she seemed to be a very healthy woman for her age, without signs of thyrotoxicosis or of myxoedema. In the neck was a large hard mass, which replaced the right lobe and caused stridor. The regional lymph nodes were palpable. A diagnosis of carcinoma was made and she was admitted to hospital. The radio-iodine uptake was found to be normal. X-rays showed deflection of both the trachea and the oesophagus to the left. At operation, the right lobe had the usual appearance of carcinoma. The growth had spread behind the trachea, which it had invaded, was fixed to the oesophagus, and extended into the mediastinum. Total thyroidectomy was impossible, but as much as possible was removed. Microscopically (Fig. 2) foci of reticulum cell sarcoma were seen proliferating in a lymphadenoid goitre. Autoradiograms showed no uptake in the tumour. There was no evidence of malignancy in the neighbouring lymph nodes and at the time of going to press there is no evidence of sarcoma elsewhere. She has been treated by radiation and remains well 15 months after operation.

Case III.

A 59-year-old man attended New End Hospital on March 16 1954 complaining of a hoarse voice for two years. A diagnosis of chronic laryngitis had been made at another hospital. Ten weeks earlier he had noticed a swelling in the neck which had become visible to his friends in the last three weeks. He was conscious of a discharge in the back of his throat and had a feeling of constriction and a choking cough at night. He had lost 7 lb. in weight. On examination he was found to be a healthy looking man with a swelling in the midline of his neck. Both lobes of the thyroid were slightly enlarged and hard. There were several firm lymph nodes palpable in the posterior triangles on both sides. The uptake of radioiodine was normal. A diagnosis of carcinoma was made and he was admitted to hospital. At operation, the thyroid was found to be adherent to the strap muscles. The neighbouring lymph nodes were soft and fleshy. A total thyroidectomy was performed. Microscopically, the tumour consisted of a reticulum cell sarcoma (Fig. 3), which was present also in the lymph nodes. The autoradiograms showed no uptake in the tumour tissue. He has been treated with X-rays and remains well 14 months later.

Case IV.

Recently a fourth case has been seen at New End Hospital, that of a woman of 80 who had developed, in the preceding three months, a swelling in the region of the thyroid gland. There were no other symptoms. The swelling was found on examination to be a collection of three stony hard nodules the size of walnuts, occupying the left lobe and the isthmus of the gland. The uptake and excretion of ^{131}I were normal but the former was almost entirely concentrated in the right lobe, a finding later confirmed by the autoradiogram. A diagnosis of carcinoma was made. At operation (Mr. J. E. Piercy) on June 17 1955 the left lobe and isthmus were replaced by " craggy " growth which appeared to be carcinomatous. A total thyroidectomy was performed. The patient made an uninterrupted recovery. Histological examination (Fig. 4) disclosed a reticulum cell sarcoma of the thyroid

invading surrounding muscles. There was no evidence of regional node involvement. X-ray therapy has been begun.

In contrast with these four cases, in which there is good reason to believe that the disorder was confined to the thyroid, three other cases of reticulum cell sarcoma involving the thyroid have been observed.

Case V.

A 25-year-old woman was admitted to Garfield Memorial Hospital because of dyspnoea of six weeks' duration. The isthmus and a portion of one lobe of the thyroid were removed, and pathological examination (Fig. 5) revealed reticulum cell sarcoma. Since physical and roentgen examination at that time were negative otherwise, this was considered to be a case of primary reticulum cell sarcoma of the thyroid gland. The patient received external irradiation over the anterior and lateral neck. Four months later, a 3 cm. nodule which appeared in the left breast was removed and proved to be reticulum cell sarcoma. Roentgenograms of the chest at that time revealed a widened mediastinum suggestive of lymphoma. This is not included as a case of primary thyroid lymphoma because the rapid and widespread involvement indicated that the disease probably originated simultaneously in multiple foci.

Case VI.

A woman of 47 attended New End Hospital with an enormous growth involving all the structures in the neck and extending downward into the mediastinum. Removal of the growth was found to be impossible. She died soon afterwards and was found at necropsy to have extensive involvement of the lungs, the pericardium and the head of the pancreas. Histologically, the growth was reticulum cell sarcoma (Fig. 6).

Case VII.

Another woman, a Hungarian of 60 years of age, was referred to New End Hospital with a story of a swelling of the neck present for two months. There were no other symptoms and her weight had been increasing gradually. She was found to have a nodular enlargement of the right lobe of her thyroid and, in addition, a mass in the right supraclavicular fossa extending below the clavicle. The mass did not move when she swallowed. X-rays showed no enlargement of the mediastinal glands. A tentative diagnosis was made of carcinoma of the thyroid with metastases in the lymph nodes. The mass and the entire right lobe of the

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EXPLANATION OF PLATES.

FIG. 1.—Case I. Low power photomicrograph of reticulum cell sarcoma of the thyroid gland, showing the uniform cell pattern. × 150.

FIG. 2.—Case II.

FIG. 3.—Case III.

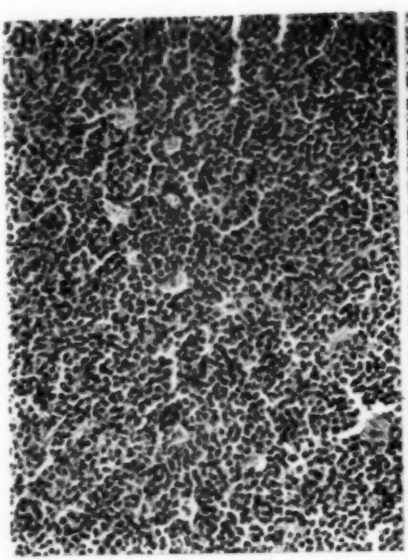
FIG. 4.—Case IV.

FIG. 5.—Case V. Reticulum cell sarcoma of the thyroid gland. × 380.

FIG. 6.—Case VI.

FIG. 7.—Case VII.

FIG. 8.—Undifferentiated carcinoma of the thyroid gland, small cell type. × 380.



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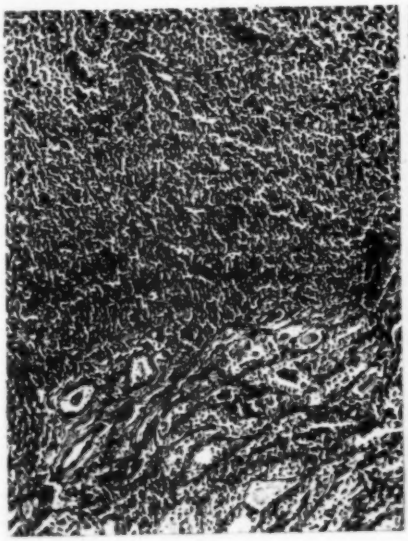

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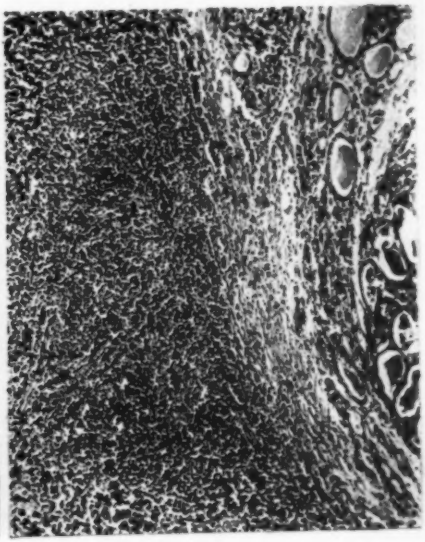

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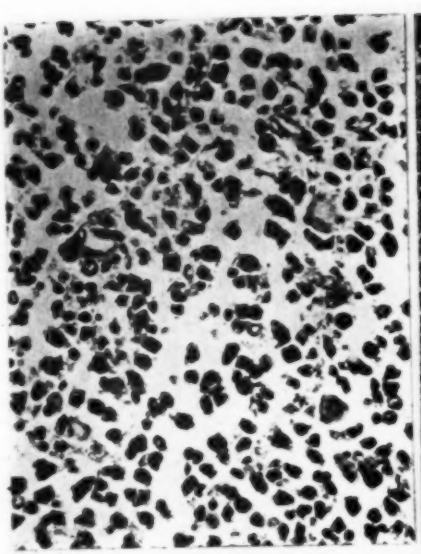

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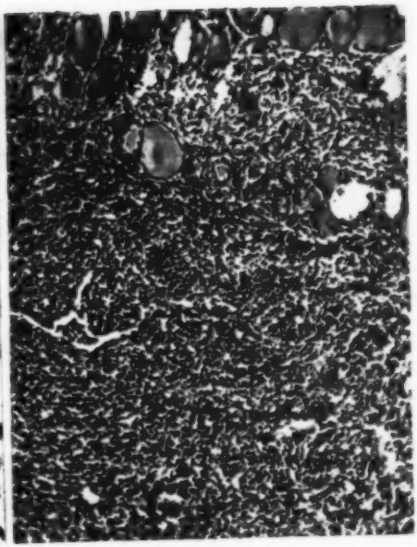

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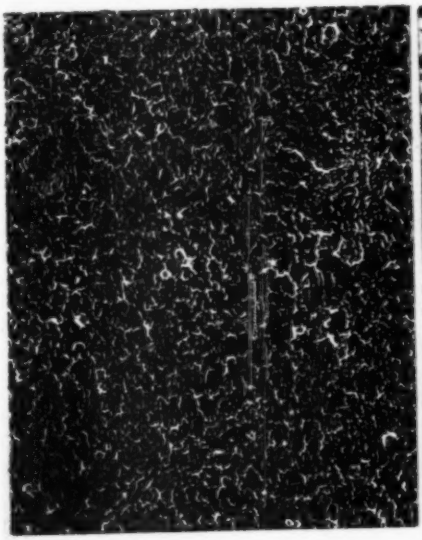

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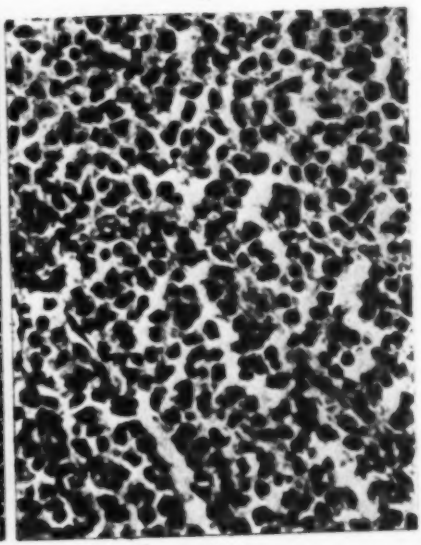

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thyroid were removed. The former was found to consist of reticulum cell sarcoma of lymphatic nodes (Fig. 7) but the thyroid showed no evidence of growth, the only microscopical abnormality being trabecular fibrosis. There was slight lymphoid infiltration of the gland. She was treated with X-rays and three years later is well in every way.,

DISCUSSION.

Doubt has been expressed that reticulum cell sarcoma can arise primarily in the thyroid gland. This is understandable since the lymphomata are usually first manifested in one or more groups of lymph nodes and nearly always appear to be multicentric in origin. Furthermore, the thyroid gland is not an organ in which a large number of reticulum cells is normally found.

Reticulum cell sarcoma probably begins in the thyroid gland as an overgrowth of the reticulum cells normally found in the interstices of the reticulum fibres which support small collections of lymphocytes. Both the aggregates of lymphocytes and the accompanying reticulum cells are potential sites of origin for lymphomata. Such collections of cells occur in at least 10 per cent of all normal adult thyroid glands (Rice, 1932) and in the great majority of glands removed for thyrotoxicosis. Struma lymphomatosa, reticulum cell sarcoma and lymphosarcoma occur with far greater frequency in females. Because of this and because of the histological similarities which exist, a relationship between struma lymphomatosa and lymphoma of the thyroid has been suggested. Such a possibility has been discussed in detail by many writers, but, except for one report (Kendrick, 1949), no definite relationship has been claimed. In our Cases I, III and IV there was no clinical or histological evidence of antecedent struma lymphomatosa in the involved lobe, and only a few small collections of lymphocytes were found in the contralateral lobe. In Case II, however, the sarcoma appears to have developed in a struma lymphomatosa of long standing.

It is not unusual to find the thyroid gland involved in generalised reticulum cell sarcoma, and when the neck organs are infiltrated widely it may be impossible to determine if the disease was multicentric in origin or if it arose as primarily reticulum cell sarcoma of the thyroid. Examples of this are Cases V, VI and VII. Possibly some of the cases reported previously as reticulum cell sarcoma primarily in the thyroid rightfully belong in a category with these cases as generalized reticulum cell sarcoma exhibiting unusual initial manifestations. The following cases, however, have been reported as primary reticulum cell sarcoma of the thyroid gland.

Ambo (1937) and Vaux (1937) each described a case of reticulum cell sarcoma apparently arising in the thyroid gland. Welti and Huguenin (1938) mentioned two cases in their 1938 series of malignant thyroid tumours. In 1949, Kellett and Sutherland reviewed the literature on reticulum cell sarcoma of the thyroid, sometimes called reticulosarcoma in England. They presented data on five new cases and discussed the histogenesis of the disease and the possible relationship between struma lymphomatosa and reticulum cell sarcoma. In the same year two cases of thyroid reticulum cell sarcoma were reported by Dean (1949), two by Cope *et al.* (1949), and one by Jackson (1949). Two years later Dick and Kellett (1951) published the autopsy findings on two patients, both of whom had intestinal metastases in addition to residual reticulum cell sarcoma in the thyroid gland. Scott (1952) studied two patients with this disease, one of whom had involvement

of the stomach. He discussed the difficulty in distinguishing reticulum cell sarcoma from certain types of anaplastic carcinoma and emphasized the fact that reticulum stains do not facilitate the differentiation. McClintock (1951) cited one additional case of reticulum cell sarcoma of the thyroid gland. The recent paper by Brewer and Orr (1953) included case histories of ten pateints with "struma reticulosa". This noncommittal term was introduced to designate a disease histologically similar to reticulum cell sarcoma but different because of the high survival rate. Four of their ten patients were living and well after a considerable period of time. The photomicrographs of the cases of Brewer and Orr do not completely exclude the possibility that carcinoma and reticulum cell sarcoma have been grouped together under the heading of struma reticulosa.

Reticulum cell sarcoma is rare but probably not as uncommon as is indicated by the limited literature on the subject. Differences in pathologic interpretation and the reluctance of American pathologists to consider the possibility of thyroid sarcoma appear to be the chief reasons why so few cases of this disease are found in the literature. Including all the cases of Brewer and Orr (1953), 70 per cent of the known cases were found in England. Two were reported from France, one from Germany, and five from the United States. This unusual geographic distribution of cases is probably only apparent and merely represents differences in diagnostic criteria. It seemed probable that some of the cases of undifferentiated thyroid carcinoma—frequently referred to as small cell carcinoma in the United States (Fig. 8)—would be diagnosed in England as reticulosarcoma or struma reticulosa. This probability was confirmed when we sent slides of our Cases I and V of reticulum cell sarcoma and of two cases diagnosed as small cell carcinoma to Brewer, Orr, Sutherland and Kellett in England for their interpretation. Their opinions regarding the slides were not unanimous but, collectively, they strongly favoured the diagnosis of reticulosarcoma over carcinoma in all of the cases. Slides on eleven cases, reported as reticulosarcoma in England and kindly sent to one of us (T. W.) were studied by several pathologists in the United States. Unanimous agreement was not reached on any case. Each case was considered by at least one pathologist to be reticulum cell sarcoma, but the most common diagnosis was carcinoma. This experience in exchanging slides demonstrated the difficulty in distinguishing histologically between sarcoma and carcinoma of the thyroid gland.

In many cases the final diagnosis must be determined by the physical findings and the course of the disease. The age and sex of the patient is of no diagnostic help for both diseases occur predominantly in elderly women, but other clinical features may be helpful. The response of the tumour to irradiation furnishes important circumstantial evidence in the differential diagnosis because small cell thyroid carcinoma generally is considered to be relatively radioresistant and reticulum cell sarcoma radiosensitive. The site of secondary disease also aids in determining the final diagnosis. Reticulum cell sarcoma, regardless of where it is first manifested, usually becomes generalized and involves groups of lymph nodes, the liver and spleen, whereas thyroid carcinoma invades the local structures of the neck and produces metastases in the cervical lymph nodes, the parenchyma of the lung and the skeleton. This is true with the exception of the recent reports of reticulum cell sarcoma of the thyroid from England. Of the eight English cases in which autopsy findings were available, seven showed metastases in the gastro-intestinal tract, and in four of these the metastases in the gastro-intestinal tract were the only ones found. These included the two cases of Brewer and Orr (1953),

the two cases of Dick and Kellett (1951), Scott's (1952) case, and the unpublished cases of Goldie (personal communication).

Although the prognosis of patients with reticulum cell sarcoma of the thyroid is generally poor, the series reviewed here suggests that the outcome is influenced greatly by early detection and prompt therapy. Analysis of the submitted clinical data on the reported cases of reticulum cell sarcoma of the thyroid shows that the surviving patients were those who had localized, resectable tumour of short duration and received adequate surgical and irradiation therapy. This analysis confirms the findings of Gall (1943), Craver (1948), Hellwig (1947) and others who have shown that localized lymphoma, when discovered early, can be controlled sometimes by aggressive surgery and adequate irradiation therapy. In view of the extreme difficulty in the clinical and histological differentiation between reticulum cell sarcoma and small cell carcinoma in the thyroid gland, it would seem advisable to employ radical surgery and irradiation in all cases of either disease.

SUMMARY.

Four cases of primary reticulum cell sarcoma of the thyroid gland are presented. Three patients presenting as such are described but considered to be examples of generalized reticulum cell sarcoma.

The literature on reticulum cell sarcoma of the thyroid is reviewed and the difficulty in diagnosis is discussed.

Evidence is presented to indicate that the disease can sometimes be controlled when the diagnosis is established early and when surgical and irradiation therapy is adequate.

We wish to express thanks to Dr. Calvin T. Klopp and Dr. Paul S. Putzki for the data on Cases I and V in this report, and to Mr. J. E. Piercy who operated on Cases II, III, IV, VI and VII ; and to Dr. A. B. Bratton and his staff for their opinions on the histology of the New End Hospital cases.

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GRANULOSA CELL TUMOURS IN INTRASPLENIC OVARIAN GRAFTS, WITH INTRAHEPATIC METASTASES, IN GUINEA-PIGS AT FIVE YEARS AFTER GRAFTING.

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EVIDENCE has been given in previous papers (Lipschutz, Ponce de León, Woywood and Gay, 1946; Iglesias, Mardones and Lipschutz, 1953*a*) that luteomata develop in intrasplenic autografts in the castrated guinea-pig; in experiments lasting 10 to 36 months these luteomata reached an incidence of about 60 per cent. They may replace the ovary completely ("exclusive" luteoma); they attain sometimes an enormous size. They infiltrate the spleen. They are not transplantable. On the contrary, in other rodents granulosa cell tumours prevail as shown as early as 1944 by the work of Biskind and others in the rat, by Li, Gardner, Furth and others in mice (Gardner, 1953; also still unpublished work of Mardones). In mice metastases may occur though rarely (Li, 1948).

Indeed, one may find in intrasplenic grafts in guinea-pigs nodules or cords of non-luteinized cells deriving from follicles or from the stroma; similar nodules or cords may constitute an important part of the latter. But we did not feel sure whether these nodules deserve the name of a thecoma or granulosa cell tumour. Though so many massive exclusive luteomata developed in our experiments when continued for as long as three years, till recently we had never seen massive or exclusive granulosa cell tumours in guinea-pigs and we never had seen metastasis.

It seemed reasonable to ask (Iglesias, Lipschutz and Mardones, 1950) whether the differential behaviour of the various rodents had to be explained as due to a difference of the time necessary for the induction of metastasizing ovarian tumours according to the species. However, since there was no experimental evidence of this, we became rather inclined to interpret the mentioned differential behaviour as an example of a genetical refractoriness to a certain type of neoplasia. But when examining lately a series of guinea-pigs necropsied almost five years after grafting the ovary into the spleen, we found an exclusive and metastasizing granulosa-cell tumour and a mixed luteoma-granulosa-cell tumour. This finding gives for the first time full evidence that the genetical difference as to neoplastic growth in the given species must be expressed not in terms of refractoriness but of differential time of evolution of the respective type of neoplasia. Needless to say that this opens a host of fundamental problems of evolutional tumourigenic dynamics in general. These two cases of granulosa cell tumours shall be described in the present paper.

The animals belonged to a series in which pellets containing very small amounts of oestradiol were implanted with the purpose of studying quantitative aspects of the inhibitory action of oestrogen on intrasplenic ovarian tumourigenesis, in continuation of former work in this field (Iglesias, Mardones and Lipschutz,

1953*b*). However, the long duration of the present experiments makes it unnecessary to deal here with this special aspect. We have found (unpublished work) that oestrogenic action of similar pellets in castrated guinea-pigs diminishes in time. The vagina which had opened closes again ; the nipples which had grown diminish in size. We do not yet know why oestrogenic action ceases ; but at about two years after implantation of the pellet containing but 0·1 to 3·0 per cent of oestradiol mixed with cholesterol, diminution of oestrogenic action on the vagina and nipples was general. It is reasonable to suppose that the control of the hypophysis by the circulating oestrogen also relaxes at that time and that the gorgeous evolution of luteomatous growth and of granulosa cell tumours in our animals took place in the course of the additional two to three years when production or delivery of gonadotrophic hormones was no more under the control of oestrogen. But both the incidence of luteomata, which was smaller than without oestrogen, and the smaller size of some of these growths apparently still denounce some inhibitory action of oestrogen in these experiments even when lasting as much as almost five years.

RESULTS.

A. *Incidence and Description of Tumours.*

The material is summarized in Tables I and II.

There were tumours in 7 out of 17 animals. The incidence is somewhat smaller than in our former series without oestrogen (Table I in Iglesias, Mardones and Lipschutz, 1953*a*). There were 3 exclusive luteomata (No. 232, 145, 47) ; no follicular structures and no individual corpora lutea were present besides the masses of lutein cells. The other two luteomata were of rather smaller size, especially No. 116 ; but even in this case luteomatous cords were infiltrating the spleen.

B. *Granulosa Cell Tumours.*

As mentioned there were two cases with granulosa cell tumours.

(1) (Table II, No. 56). The greatest diameter of the tumour was less than 1 cm. Already at low augmentation (Fig. 1) two different kinds of nodules could be distinguished : clear and dark ones, in general separated from one another by fibrous strands. Part of the clear nodules are composed of large cells with a vacuolated protoplasm. These cells are coincident, as to structure, with those of the corpus luteum. The darker nodules are constituted of smaller cells of the granulosa cell type. The growth is thus a mixed tumour consisting both of luteomatous and granulosa cell tissue (Fig. 2). However, granulosa cell tissue greatly prevailed (Fig. 3) : probably no less than four-fifths, or more, of the tumour were nodules or large areas of the granulosa-cell type. No follicular structures were present ; there were indeed in some granulosa cell nodules small cavities which one may suspect of being the remnants of follicular structures. In some areas the granulosa cells evidently underwent a transformation ; their protoplasm became vacuolated, and several of the clear nodules in Fig. 1 consist of these vacuolated granulosa cells. But it is remarkable that the vacuolated granulosa cells did not take the aspect of lutein cells ; there was a definite difference between both even when vacuolated. In some places nodules of granulosa cells are in immediate contact with lutein cells. A nodule of granulosa cells may even be completely surrounded by lutein cells.

TABLE I.—*Seventeen Castrated Guinea-pigs with Intrasplenic Ovarian Grafts.*

Duration of experiment. Months.	Number of animals. Total.	Animals with		
		HF.	Luteoma.	Granulosa-cell tumour.
56–58*	17	6	5†	2‡

* One animal only 48 months : No. 232 in Table II.
† Out of these, 3 were exclusive luteomata : No. 232, 145, 47 in Table II.
‡ One of these was a mixed tumour : No. 56 in Table II.

TABLE II.—*Description of 7 Tumours and of their Functional Condition.*

CLI. No.	Duration (days).	Ovarian tumours (mm.).	Genital region : Clitoris. Corpora cavernosa (mm.).	Horny styles (mm.).	Uterine weight (g.).	Uterine epithelium.	Mammary gland.
232	1441	Exclusive luteoma 13 × 8	3	1·5	1·3	Metaplasia Cystic hyperplasia	—
145	1683	Exclusive luteoma 10 × 5 Large " Brenner " 4 × 2	2	2	1·6	Metaplasia Cystic hyperplasia Polyps	Pregn.
47	1764	Exclusive luteoma 13 × 8	3	2	2·7	Metaplasia Cystic hyperplasia Polyps	Pregn
116	1681	Luteoma, infiltrative 5 × 3	—	—	1·3	Cylindrical epithelium	—
61	1749	Luteoma, infiltrative 8 × 6	—	—	1·0	Cubical and cylindrical epithelium Cystic hyperplasia	0
56	1765	Mixed : luteoma *granulosa cell* 7 × 6	—	—	0·7	Cubical epithelium Cystic hyperplasia	—
129	1666	*Granulosa cell* Fig. 4	4	1	26·0	" Adenocarcinoma " see text and Fig. 11–13	0

In right-hand column — = glandular tissue not found at necropsy and not examined microscopically ; 0 = glandular tissue not found at necropsy and neither at microscopical examination.

(2) (Table II, No. 129). A formidable growth was found at necropsy in the splenic region (Fig. 4). The weight of the spleen together with the growth was 51 g., of which only about 1 g. corresponds to the spleen. At incision compact tissue was seen intermingled with what seemed to be necrotic and haemorrhagic masses. The colour of the tumour was reddish-grey. Irregularities of the same colour were to be seen on the surface of the liver ; it was the same when incisions were made. At microscopical examination the growth was shown to consist of compact masses of cells of the granulosa type (Fig. 5) adjoining necrotic or haemorrhagic zones, or zones of conjunctive tissue, sometimes fibrous but more often loose and oedematous. At many places the granulosa cells are intermingled

with a variable number of small cells, probably leucocytes. The liver was filled with nodules which were an exact replica of the tumour (Fig. 6, 7); the microphotographs of both were identical (Fig. 6 and 8). There was not the slightest doubt about the intrahepatic nodules being metastases of the tumour

c. *Growth of Wolffian structures.*

As in former work with intrasplenic ovarian autografts in castrated guinea-pigs atypical growth of Wolffian structures was very prominent also in the present series of long duration.

EXPLANATION OF PLATES.

Fig. 1, 2, 3, *are from the same animal.*

FIG. 1.—Intrasplenic ovarian graft; 1765 days (CLI.56). No follicular structures and no individual corpora lutea. The tumour consists of luteomatous nodules and of nodules of granulosa cells. The latter prevail. × 4.

FIG. 2.—Mixed part of the tumour. At the bottom, cords of luteal cells separated from the spleen by a fibrous capsule. At the top luteomatous cords in immediate contact with large nodule of granulosa cells. × 100.

FIG. 3.—Nodule of granulosa cells. Circular disposition of the cells at various places. × 300.

Figs. 4 *to* 8 *are of tumour and metastases of the same animal.*

FIG. 4.—Intrasplenic ovarian graft; 1666 days (CLI. 129). Enormous tumour; to the left adhesion of epiploon. × 4/5.

FIG. 5.—Tumour; compact mass of granulosa cells. × 200.

FIG. 6.—Large intrahepatic metastasis. × 15.

FIG. 7.—Small intrahepatic metastasis. × 100.

FIG. 8.—Compact mass of granulosa cells of large metastasis. Many leucocytes. × 200.

Fig. 9 *to* 13 *are from the uterus of the same animal as Fig.* 4 *to* 8.

FIG. 9.—Large uterine nodule. Note unilateral position of the latter which is typical of the oestrogen-induced nodule. Cystic enlargement of glands filling the uterine cavity. × 4.

FIG. 10.—Fibromyoadenomatous part of the nodule. Enlarged glands in the longitudinal layer and reaching the subserous layer of fibromuscular tissue which can be seen in uteri under the prolonged action of oestrogen. × 70.

FIGS. 11, 12 and 13.—Epithelial cords in the fibromyoadenomatous nodule. "Adenocarcinoma." × 100.

Fig. 14 *and* 15 *are of the same animal.*

FIG. 14.—A. Distended uterine wall surrounding fibroadenomatous polyps. Note the irregular disposition of the distended glands.

B. Uterus of the same animal; uterus not distended. Intrasplenic ovarian graft, 1762 days (CLI. 50). There was no ovarian tumour, though indeed cords of luteinized cells, an haemorrhagic follicle, Graafian follicles and corpora lutea were present. The small uterine weight, the castrate condition of the endometrium and the undeveloped mammary gland make it clear that the fibroadenomatous polyp was produced long before necropsy. × 16.

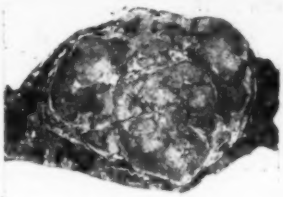
FIG. 15.—The same polyp as in Fig. 14A. Note metaplasia of endometrium and glands. × 100.

FIG. 16.—A. Ovarian intrasplenic graft, 360 days after grafting (CXXVI. 96). The last 90 days progesterone was administered by absorption from pellet. A considerable part of the graft is occupied by "follicular clusters." Granulosa cell tumour? × 35.

B and C. Details of the same. × 200.

FIG. 17.—A. Ovarian intrasplenic graft, 798 days after grafting (CLI. 68). A considerable part of the ovary is occupied by "follicular clusters." Granulosa cell tumour? A pellet containing 1 per cent of oestrogen was implanted. No luteoma was present. × 35.

B. Details of the same. × 200.



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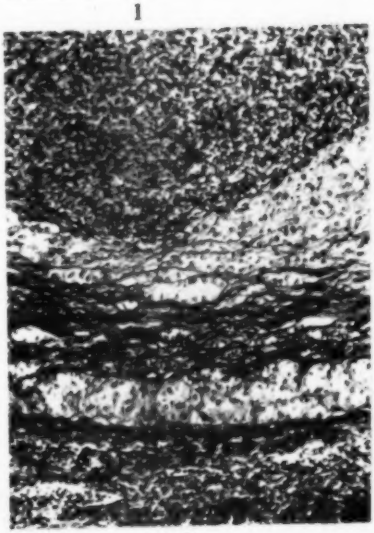

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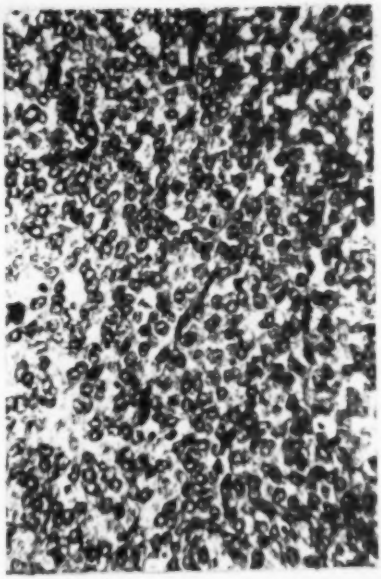

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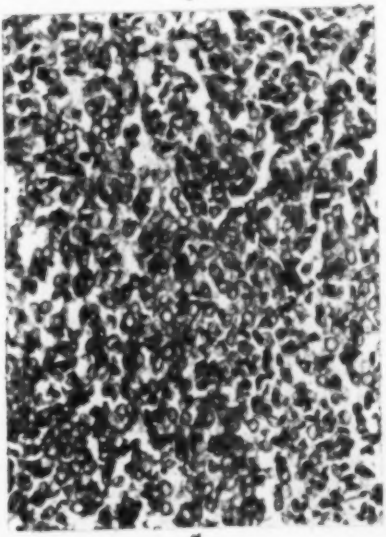

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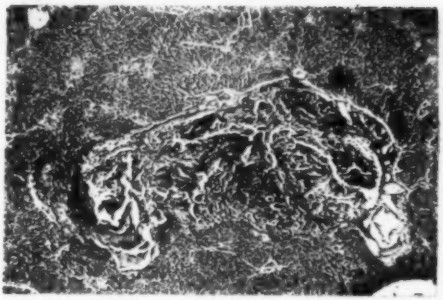
Mardones, Iglesias and Lipschutz.



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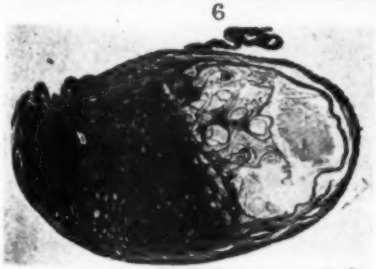

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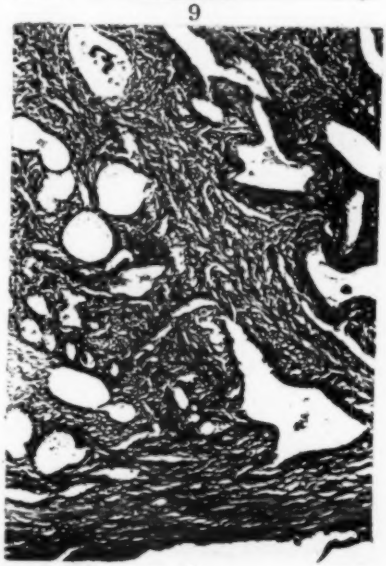

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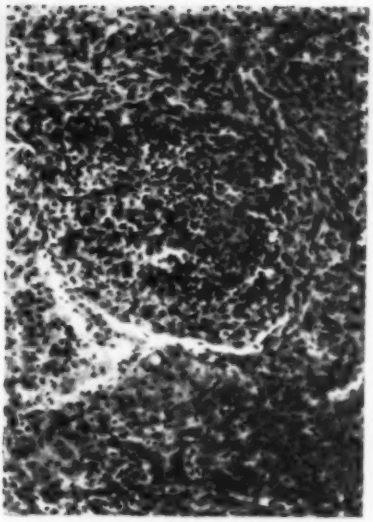

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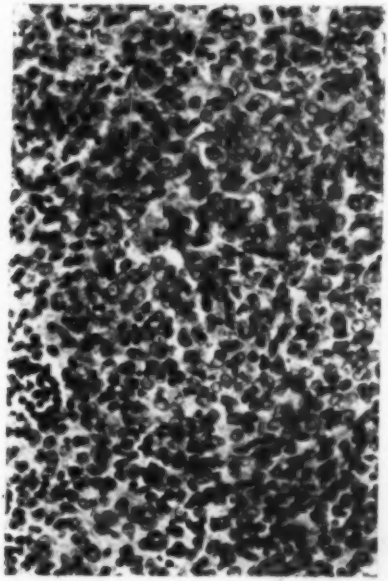

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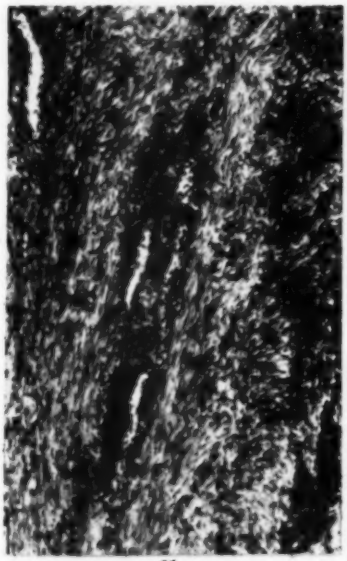

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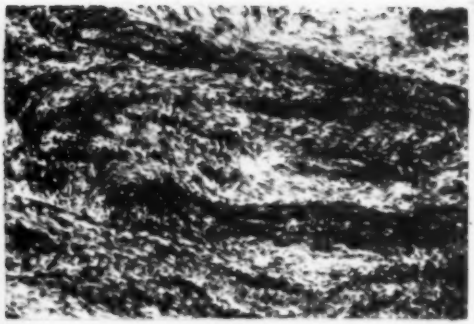

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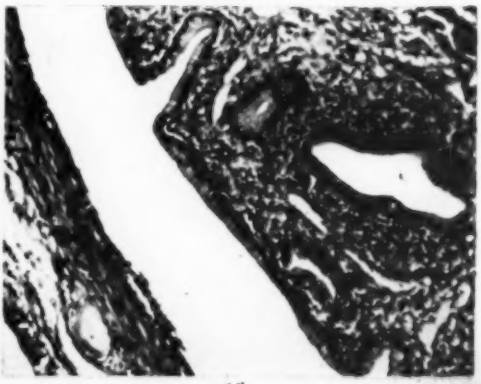
14B

14A



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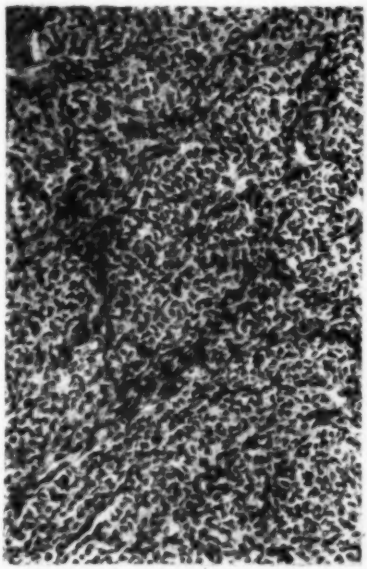
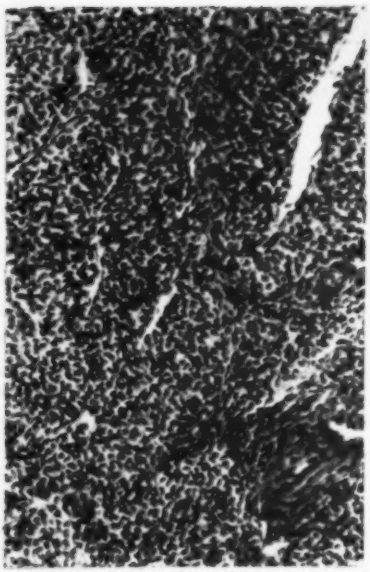


16B



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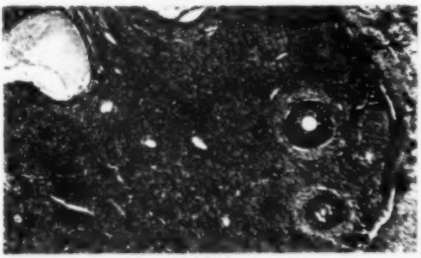
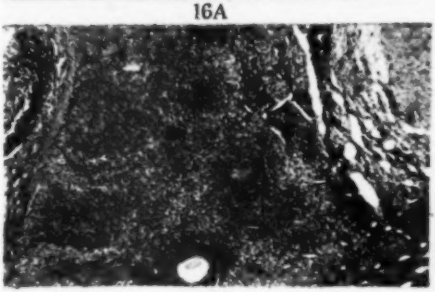
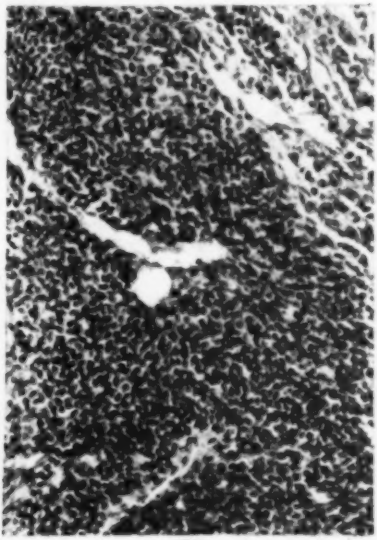


16A



17A



17B

Cysts of variable size reaching in some cases about 2 cm. in diameter (Table II, No. 47) were present in most of the animals.

Fibroadenomatous nodules as described in former papers (Lipschutz, 1950; Iglesias, Mardones, Bruzzone and Lipschutz, 1953; Iglesias, Mardones and Lipschutz, 1953*a*), which in some of their aspects resembled Brenner tumours in women, were found in several animals. In 2 cases they were larger than in any animal before. These tiny nodules seem all to be of Wolffian origin (Bruzzone and Lipschutz, 1954).

No Wolffian structures or Wolffian growth were found associated with the two granulosa-cell tumours recorded above. But this is most probably purely accidental.

We may mention that in the meantime various papers referring to Brenner tumours in women have been published (Greene, 1952; Kerpe, Black and Speer, 1952; Teoh, 1953). The histogenesis of this growth has been amply discussed in the course of years; the site of its origin has been placed in the germinal epithelium, in the ovarian stroma, in the rete and even in the Müllerian epithelium. However, according to Teoh (1953) and Willis (1953, p. 490) the Brenner tumours in women have the same histogenesis as granulosa cell tumours. If this were true for all Brenner tumours, the fibroadenomatous nodules originating in our intrasplenic grafts and whose Wolffian origin can scarcely be doubted could not any longer be compared to Brenner tumours in women.

D. *The Functional Condition of Luteomata and Granulosa-cell Tumours.*

(1) *Androgenic action of ovarian tumours.*

As shown in our former paper (Iglesias, Mardones and Lipschutz (1953*b*) the exclusive luteoma originating in the intrasplenic graft may be functional, causing both feminization (growth of the uterus and mammary glands) and masculinization (growth of the corpora cavernosa of the clitoris and of the horny styles (Bruzzone and Lipschutz, 1953)). In the present series of greater duration androgenic action was evident in all the three animals with exclusive luteoma (Table II). The corpora cavernosa reached a length of up to 3 mm., and the horny styles of up to 2 mm. It was evident that the luteoma was responsible for this masculinization by producing androgens or stimulating its production in the suprarenal cortex; the first is the more probable.

A very notable degree of masculinization was reached also in one of the two animals with a granulosa cell tumour (Table II, No. 129): the clitoris was 4 mm. long and the horny styles 1 mm. It would be idle to discuss the question of where the androgen originated in the present case. The genital region of the second animal with the granulosa cell, or mixed, tumour (Table II, No. 56) was normal.

(2) *Oestrogenic action of ovarian tumours.*

The functional condition of the granulosa cell tumour is of an especial interest. Spontaneous ovarian tumours in rodents are known to be functional (mice: Gardner, Strong and Smith, 1936; Strong, Gardner and Hill, 1937; rats: Iglesias, Sternberg and Segaloff, 1950; Iglesias, and Mardones, 1954; Iglesias, 1954).

In our animal with the smaller granulosa cell, or mixed, tumour (No. 56) oestrogen was present but in quantities probably not sufficient to cause an oestrous

condition: the cells of the endometrium were cubical; uterine weight was probably somewhat less than normal (only 0·7 g., as against 1·3, 1·6 and 2·7 g. in animals with exclusive luteoma); the vaginal mucosa indeed showed proliferation of the basal cells with vacuolated cells nearer the surface; but the mammary glands could not be visualised at necropsy. However, in the course of the 58 months which this experiment lasted there were phases in which the oestrogen concentration was sufficient to cause a cystic glandular hyperplasia of the endometrium.

A picture quite different was offered by the animal with the large pure granulosa cell tumour (No. 129). The uterus was monstrous both as to its weight which was 26 g., and as to its shape. The uterine horns and also the basal part of the uterus had been transformed into large sacs distended by a liquid content; but at the proximal end of the right horn a hard growth was found which was about 1 cm. in diameter (Fig. 9). The epithelium of the endometrium was less than cubical; at some places solitary cystic glands were to be seen whose epithelium was likewise less than cubical. Of especial interest is the above-mentioned hard growth. It consists of fibromuscular tissue of the uterine wall with proliferated uterine glands embedded in it. Many of these glands were distended, especially those located near to the uterine cavity; but distended glands of a minor diameter may be found also reaching, or penetrating into, the longitudinal layer of the myometrium (Fig. 10). So far the picture is coincident with that which is obtained in the guinea-pig with the prolonged administration of small quantities of oestrogen as described by the workers of this laboratory (Riesco, 1947; Lipschutz, 1950, pp. 82, 83; Iglesias, Mardones and Lipschutz, 1953*b*). However, there was also another aspect of fundamental interest: many glands apparently showed no cavity at all. These glands appear in long cords between the fibrous or fibromuscular strands, or they form nodules of variable size (Fig. 11, 12, 13). Whereas among the more solitary glands one may sometimes find a well developed glandular epithelium though not of the high cylindrical type, the cells of these cords or nodules are poor in protoplasm. On the whole it is a picture not simply of adenofibroma or adenomyosis but most probably of adenocarcinoma.

One may ask whether the described uterine adenofibromyomatous and adenocarcinomatous growth was due to oestrogen absorbed from the 0·5 per cent oestradiol pellet (0·5 of oestradiol mixed with 99·5 of cholesterol) or to oestrogen produced by the tumour. However, from our experience with 29 castrated guinea-pigs which were necropsied up to 1239 days after the implantation of a 0·5 per cent oestradiol pellet, we may say that never was a uterus seen which would have been similar to that in our animal with the granulosa cell tumour. This is why there cannot be any doubt that the tumoural growth of the uterus in our animal No. 129 was due to the functional condition of the ovarian tumour.

There are, on the other hand, two important facts: (1) at the end of the experiment the oestradiol pellet was no longer present in the animal—it was not found at necropsy; (2) the oestrogenic action of the granulosa-cell tumour also was nil when the animal died: the length of the nipples, which was at the beginning of about 8 mm., had diminished to 3 or 4 mm.; the mammary glands were of poor development and no glandular lobules were found at microscopical examination; the vaginal mucosa was of the castrate type. There is thus full evidence that oestrogen had long since ceased to be available in the general circulation.

The uterine epithelial growth, partly " adenofibromyoma ", partly " adenocarcinoma ", as conditioned by the prolonged action of oestrogen produced in the functional granulosa-cell tumour, did not regress when oestrogen was no more available. The epithelial growth was no longer dependent on oestrogen and as to this it reached " autonomy ". Examples of such an autonomous condition of oestrogen-induced epithelial growth in the guinea-pig have been given in former papers from this laboratory (Lipschutz, Iglesias and Vargas, 1939 ; Lipschutz, 1950, ch. 8 ; Bruzzone and Lipschutz, 1954).

When discussing the problem of autonomy reached in a certain phase of neoplastic evolution in the guinea-pig under the influence of oestrogen it is of interest to take notice of a growth as pictured in Fig. 14, from one of the animals of the present series but without ovarian tumour (Table I). The weight of the uterus was of only 0·7 g. ; the endometrium was of the castrate type ; the mammary gland could not be visualised at necropsy ; there was a slight oestrogenic action on the vaginal mucosa. In one of the uterine horns a growth about 3·5 mm. in diameter and attached to the uterine wall filled the whole cavity. The growth consisted of proliferated and distended uterine glands embedded in an irregular manner in conjunctive tissue. The glandular epithelium was cylindrical ; in many places it was very high and metaplastic, with a clear and vacuolated protoplasm (Fig. 15). This epithelium contrasted fundamentally with that of the endometrium or of the glands in the tunica propia of the non-distended uterine horn. It is evident that oestrogen concentration in the general circulation had dropped in this animal to a level no longer sufficient to maintain the epithelium of endometrium and glands in an oestrous condition ; but this low level of oestrogen concentration was sufficient, or no longer necessary at all, for the maintenance of the uterine tumoural growth. We see that the " classical ", or " traditional " concept of neoplastic growth becoming independent from the extracellular stimulus, by which it was originally induced, cannot be dealt with simply in an alternative manner in terms of " presence " or " absence " of this stimulus ; when applied to neoplastic growth induced by oestrogen it turns out to be again a problem of differential oestrogen concentrations as discussed in a former paper with reference to other examples (Iglesias, Mardones and Lipschutz, 1953*b*).

DISCUSSION.

The evidence presented in the foregoing description, that the guinea-pig is not genetically refractory to experimental production of ovarian granulosa cell tumours, has served as an incentive for making a search for those cases in our collection of intrasplenic ovarian grafts in which doubt might arise about the nature of certain cellular nodules in experiments of shorter duration (one to two years) and whose character as granulosa cell tumours we were inclined to deny. We shall deal first with experiments in which progesterone was administered with the object of testing the antiluteinizing faculties of this steroid on luteomata.

Progesterone is known to counteract luteinization in the intrasplenic ovarian graft (Lipschutz, Iglesias, Bruzzone, Humérez and Peñaranda, 1948 ; Mardones, Bruzzone, Iglesias and Lipschutz, 1951). Consequently, when a pellet of progesterone was implanted into castrated guinea-pigs with intrasplenic ovarian grafts ten months after grafting and the progesterone was allowed to act for at least three months before necropsy, luteomata did not occur (Iglesias, Lipschutz and Mardones, 1950). No luteal cords were present in the stroma of these grafts.

Corpora lutea were in a state of degeneration. On the contrary, the graft contained large clusters of small non-luteinized cells mostly of follicular origin. These follicular clusters are often very similar to small atretic follicles. The cells are smaller than the cells of the follicular granulosa. There were also large nodules of similar cells scattered in the stroma not reminiscent of any follicular structure (Fig. 4*b* and 5 in Iglesias, Lipschutz and Mardones, 1950). But we did not feel sure whether these clusters or nodules of cells could be called granulosa cell tumours. Now, comparing these clusters or nodules obtained in older experiments in which luteinization was counteracted by the prolonged action of progesterone (Fig. 16), with the picture offered by the exclusive granulosa cell tumour (Fig. 5) or the mixed tumour (Fig. 3) in the present experiments, one might wonder whether a condition like that in Fig. 16 (coincident with Fig. 4*a* and 4*b* in Iglesias, Lipschutz and Mardones, 1950) has to be interpreted as of a granulosa cell tumour. This indeed was the opinion of various pathologists. We ourselves do not feel competent to settle the question; the pictures obtained with intrasplenic grafts and the simultaneous use of progesterone as an antiluteinizer are too similar to pictures one may see in the normal ovary also.

The fact that progesterone, by interfering in hypophysial events, prevented luteinization and production of luteomata* and that nodules suspected of being granulosa cells became under these circumstances more prominent than is the general rule in the guinea-pig would not mean that the luteoma is always but a luteinized granulosa cell tumour. The question has already been fully discussed in a previous paper (Iglesias, Mardones and Lipschutz, 1953*a*).

Nodules of non-luteinized cells of a type similar to that in Fig. 16, or in Fig. 3 and 5, have been found also, though rarely, in intrasplenic grafts when no antiluteinizer was used; but then corpora lutea or cords of lutein cells also were present. An example is given in Fig. 17, from an animal necropsied at about two years after grafting. However, here again doubt arises on account of the similarity with the picture offered by normal ovaries.

One may care to interpret all these observations in the sense that granulosa-cell tumours can appear in the intrasplenic graft in guinea-pigs as early as one or two years after grafting, especially when luteinization is inhibited by the administration of progesterone. But the fact remains unshaken that in guinea-pigs, contrary to mice, luteomata prevail in the intrasplenic graft, that production of granulosa-cell tumour is only a very rare event, and that an exclusive granulosa-cell tumour with metastasis is produced only exceptionally when the experiments last many years.

SUMMARY.

In a group of 17 guinea-pigs with intrasplenic ovarian autografts necropsied 54 to 58 months after transplantation two granulosa cell tumours appeared.

In one case the granulosa cell masses were accompanied by nodules of lutein cells; in the other case the tumour consisted only of granulosa cells and connective tissue.

In the last mentioned case multiple metastases of variable size were found in the liver.

* Prevention of *luteomata* was not obtained in mice when progesterone was administered (Li and Gardner, 1949). But this might have been due to insufficient quantities of progesterone injected once weekly whereas in our experiments with the subcutaneous implantation of pellets progesterone was allowed to act *continuously*.

The cells of the metastases were identical with those of the tumour.

It is thus fully evident that the differential behaviour of the ovarian intrasplenic autograft in mice where granulosa cell tumours prevail, on one hand, and in guinea-pigs where luteomata prevail, on the other hand, cannot be interpreted simply as refractoriness of the latter species to a given type of neoplasia, i.e. of granulosa cell tumours. The difference must be expressed not in terms of refractoriness but of differential time of evolution of the respective type of malignant neoplasia according to the species.

The metastasizing granulosa cell tumour of the guinea-pig was functional, producing both androgenic and oestrogenic actions. There was, as with exclusive luteomata, masculinization of the clitoris, a hypospadic penis being produced; there was a uterine picture reminiscent of adenocarcinoma.

We are greatly indebted to Dr. R. Barahona, Professor of Pathology of Universidad Católica de Chile, for the examination of our slides and valuable information.

Thanks are due for technical help to Mrs. Julia Peña, and to Mrs. Leopoldina Grabherr, photographer.

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FURTHER INVESTIGATIONS ON THE INTERACTION OF POLYCYCLIC HYDROCARBONS WITH EPIDERMAL CONSTITUENTS.

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MANY workers have at various times suggested that the cancer-inducing capacity of certain polycyclic hydrocarbons is connected in some way with their ability to combine or associate with cell constituents. Miller (1951) and Miller and Miller (1952) produced experimental evidence to show that a proportion of such a carcinogen applied to the skin becomes firmly bound to the epidermis of the treated area forming a protein-hydrocarbon complex from which the hydrocarbon (or closely allied derivatives) can be extracted only after complete hydrolysis of the tissue. It was suggested that the loss of some vital protein which results from such combination is a necessary stage in the evolution of the new type of cell.

It has become a commonly accepted view that the cancer inducing activity of such chemical agents must be explained in terms of a direct chemical action or combination but there is some contrary opinion and evidence (Orr, 1954; Vernoni, 1951).

In a previous paper (Woodhouse, 1954) it was shown that certain polycyclic hydrocarbons, inactive as skin carcinogens to mice, *e.g.*, perylene and dibenzacridine, also become bound to epidermal protein. Moodie, Reid and Wallick (1954) believe that their experiments indicate that the non-carcinogens are much more weakly held than the carcinogenic hydrocarbons. Heidelberger and Moldenhauser (1955) employing hydrocarbons labelled with C^{14}, also showed that phenanthrene and 1 : 2 benzanthracene, inactive, or very weak carcinogens, are bound to a much less extent than the powerful ones such as methylcholanthrene or 9 : 10 dimethylbenzanthracene (D.M.B.A.). They also found, on the other hand, that 1 : 2, 3 : 4 dibenzanthracene, a non-carcinogen, is " extensively " bound.

Thus the observations are not at present capable of giving a simple explanation of the process and further experiments are necessary to assess the significance of such combination. This may be mainly concerned in the detoxification and elimination of such substances and unconnected with, or in addition to any specific interactions that are concerned with the production of neoplastic cells.

This report describes three series of experiments designed to obtain information regarding : (1) The amount of bound hydrocarbon produced either under conditions which modify the response of the skin to carcinogenic hydrocarbons or under the influence of agents which induce epidermal changes not leading to malignancy (Experiments Series 1). (2) The persistence of free and bound hydrocarbon after a single massive application of 9 : 10 dimethyl-1 : 2-benzanthracene (Experiments Series 2). (3) The binding property of another non-carcinogenic hydrocarbon-

benztetraphene (Experiments Series 3). This compound is of interest since it is non-toxic, does not inhibit somatic cell growth like many carcinogens, but has the property of strongly inhibiting the growth of grafted tumours and some spntaneous tumours, and has been suggested as capable of modifying the antigenic structure of a specific protein complex (Green, 1954). It is highly fluorescent in organic solvents and can be easily detected in " ultra-violet " light at a dilution of 1/10μg./ml. or less in benzene or toluene.

EXPERIMENTAL.

(1) *Reagents.*

The following agents were employed in conjunction with the hydrocarbons for the reasons given.

(*a*) Croton oil : This substance has been shown to be the most powerful known co-carcinogen for mouse skin and has been extensively used in investigations on the elicitation of skin tumours. (Berenblum and Shubik, 1947, 1949 ; Salaman and Gwynn, 1951). It produces effects on mouse skin " not unlike those produced by carcinogens " (Orr, 1938). Continued applications to the skin produce a few tumours in mice (Salaman and Roe, 1953). It was employed (Ol crotonis Ang. B.P.C.) 0·5 per cent solution in acetone, 0·2 ml. per application.

(*b*) Nitrogen mustard produces, after 3–10 days, a type of epidermal hyperplasia chiefly characterized by gross irregularity in size and arrangement of cells, and a similar histological appearance in the epidermis to that produced by 9 : 10 dimethylbenzanthracene (Salaman and Roe, 1953). It was used as 0·2 per cent solution in acetone, 0·2 ml. per application.

(*c*) Urethane : This is described by Salaman and Roe (1953) as an effective "initiating principle " which causes no skin changes apparent microscopically or macroscopically, and no tumours. It was used as 20 per cent $\frac{W}{V}$ aqueous solution, 0·2 ml. per application.

(*d*) Maleic anhydride was shown by Crabtree (1945) to have, in common with some other unsaturated dibasic acids, the property of inhibiting the production of skin tumours by benzpyrene. It was used as 6 per cent aqueous solution.

(*e*) Cortisone has been found to inhibit the production of skin tumours by D.M.B.A. when applied topically, concomitantly with the carcinogen. (Engelbreth-Holm and Asboe-Hanson, 1953) (Ghadially and Green, 1954). It has a depressant effect on mititic activity in mouse epidermis (Green and Savigear, 1951 ; Bullough, 1952).

(*f*) The specimen of 3 : 4 benztetraphene ; naphtho-(2′: 1′, 1 : 2) anthracene (Clar, 1952, p. 207) used in Experiments Series 3 was kindly provided by Professor H. N. Green. It was used as 0·1 per cent solution in toluene. It was not very soluble in most organic solvents and this medium was necessary to obtain the required concentration.

(2) *Extraction and measurement of bound hydrocarbon.*

The method previously described (Woodhouse, 1954) was used for preparing the protein from the mouse skin. It is essentially that used by Miller (1951). After thorough extraction with alcohol, which has been shown to extract the unbound B.P. completely, 25 mg. samples of the dry protein were hydrolysed for

2 hours with 4N alcoholic NaOH in presence of toluene and zinc dust. The solution (referred to as the alkaline hydrolysate) was extracted several times with 10 ml. benzene.

The benzene extracts, after separation and dehydration with sodium sulphate, were combined and reduced to a suitable volume for fluorimetry.

The aqueous alkaline solution was neutralized with HCl, brought to a slightly acid pH and again extracted with benzene. This portion is referred to as the extract from the acidified hydrolysate. The estimation of the fluorescence values of the extracted hydrocarbon from both procedures was also conducted as before by comparing the extracts with appropriate dilute standard solutions of the particular hydrocarbon initially applied.

(3) *Application of Solutions.*

All solutions were applied to the backs of the animals from which the hair had been cleanly clipped, with care to avoid damage to the skin, one day prior to the first application. When observations continued over a period the hair was again removed as necessary to provide a clean tissue for chemical treatment.

Procedures in Experiments Series 1.

(1) Croton oil (followed by benzpyrene) ;
Urethane (followed by benzpyrene) ;
Nitrogen mustard (followed by benzpyrene).

Three applications of the preparing agent at 3 day intervals were made over an area of the skin somewhat greater than that subsequently removed for chemical treatment. The hydrocarbon solution was then applied for 6 successive days and the skin removed after a further 2 days. It was not deemed practical to test the effect of applications of the agents after benzpyrene treatment because this might remove or disturb the carcinogen and lead to confusion in the quantitative interpretation of the results.

(2) Urethane followed by benzpyrene ;
Maleic anhydride followed by benzpyrene.

Applications were made every other day, for 6 days followed by benzpyrene treatment and skin removed as in the other experiments of this group.

Procedures in Experiments Series 2 *and* 3.

In the experiments for observing the persistence of 9 : 10 dimethylbenzanthracene one application of 0·5 per cent solution (1000 μg.) was made and groups of 3 mice were killed at appropriate intervals. It was necessary to confine the exposure to a single application with this concentrated solution owing to its " caustic effect " on the skin. The skin of any animal which showed obvious damage was not used for extraction. Histological examination of the skin of animals treated similarly showed, however, that marked histological changes occurred during the period of the tests (personal communication from Professor Orr).

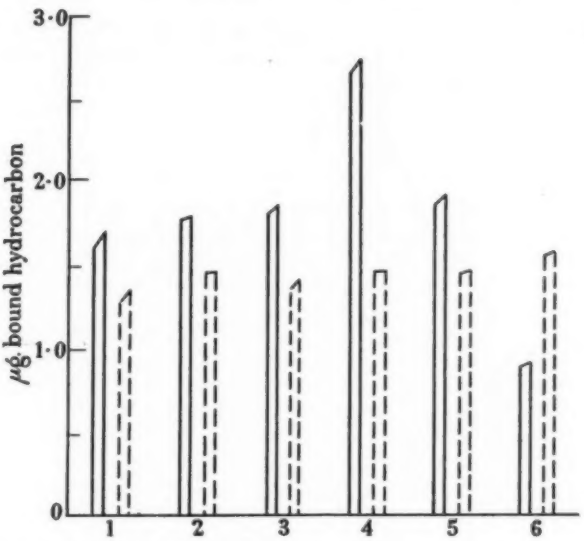

FIG. 1.—Bound Benzpyrene in 25 mg. dry epidermal protein. (1) Six applications of 0·4 per cent Benzpyrene. (2) Pretreatment of skin with Croton oil. (3) Pretreatment of skin with Urethane. (4) Pretreatment with Nitrogen mustard. (5) Pretreatment with Cortisone. (6) Pretreatment with Maleic anhydride.

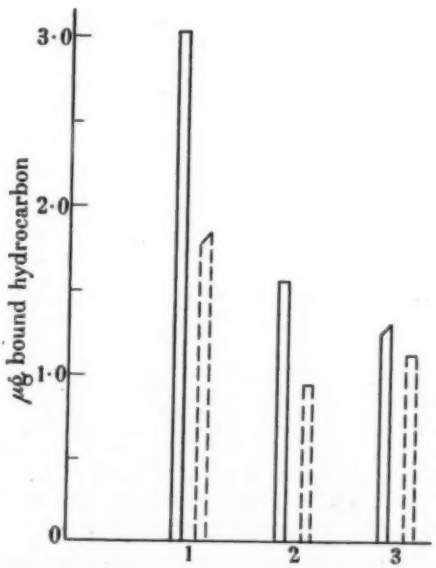

FIG. 2.—Bound hydrocarbon in 25 mg. dry epidermal protein.
———— Alkaline hydrolysate. – – – – Acidified hydrolysate.
(1) 9 : 10-dimethyl-1 : 2 : benzanthracene. (2) Benztetraphene. (3) Perylene.

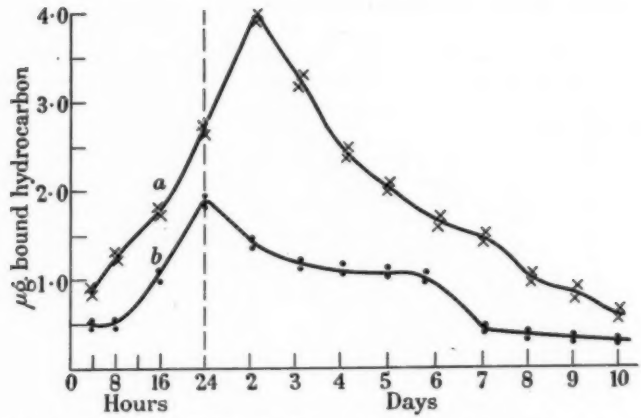

FIG. 3.—Persistence of bound D.M.B.A. in 25 mg. dry epidermal protein. One application of 1000 μg. per mouse. (*a*) Alkaline hydrolysate; (*b*) Acidified hydrolysate.

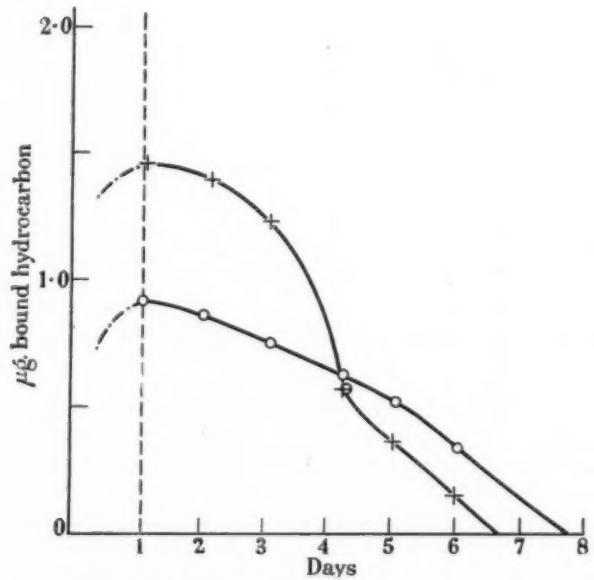

FIG. 4.—Persistence of bound 3 : 4 benztetraphene in 25 mg. dry epidermal protein. Two applications (total 200 μg. per mouse); (*a*) Alkaline hydrolysate; (*b*) Acidified hydrolysate.

Four applications of 0·2 ml. of the benztetraphene solution were made during one hour in order to apply a sufficient amount of hydrocarbon (400μg.) and the animals killed after 24 hours. For further comparison other groups of mice were given a single application of 0·2 per cent D.M.B. (400μg.) and killed after 24 hours.

RESULTS.

Fig. 1 sets out the results in the tests of Series 1 and gives the amount of bound benzpyrene (and/or metabolites) extracted from the alkaline hydrolysate from samples of dry protein and also the amounts obtained after acidifying the hydrolysates. The figures are the average values from duplicate analyses on 25 mg. samples of alcohol-extracted protein from two separate preparations (4 analyses in all). The skin from three mice was used for each preparation.

The amounts of bound benztetraphene obtained 24 hours after one application are similarly shown in Fig. 2, together with the values obtained in the parallel experiment with 0·2 per cent D M.B.A. The values from a previous test using the non-carcinogen perylene are also given.

The persistence of bound D.M.B.A. during the first 24 hours after the single massive application and up to 10 days is shown on Fig. 3 while the persistence of bound benztetraphene is shown on Fig. 4.

DISCUSSION.

The following observations can be made from the four charts.

1. In Fig. 1 two values appear to differ significantly from the others, namely the high content of the alkaline extract after the nitrogen mustard (+ benzpyrene) treatment, and the low value for the alkaline extract from the protein of the maleic anhydride treated skins. The latter is low when compared either with the amount in the companion acidified extract or with respect to the content of the other alkaline hydrolysates in this series.

2. The amounts recovered from the acidified hydrolysates of the benzpyrene-treated skin in all the other groups approximated closely to the amount found in the benzpyrene controles.

3. The bound hydrocarbon in the alkaline hydrolysate from the D.M.B.A.-treated skins was greater than that obtained with the other hydrocarbons tested (Fig. 2 and 3).

4. The amount of benztetraphene in extracts from either acid or alkaline medium was not essentially different from the amount of hydrocarbon in the analogous B.P. hydrolysates.

5. Significant amounts of bound D.M.B.A. or derivatives were present up to the 9th or 10th day (Fig. 3). At this time appreciable amounts of " free " fluorescent material also remained which could be extracted by warm alcohol. A detailed quantitative measurement of this was not undertaken as it is recognised that epidermis not exposed to hydrocarbon yields some " background " fluorescence. This is, of course, eliminated during the extraction procedures to which the tissues are subjected before they are finally hydrolysed for the bound fluorescent components.

6. Benztetraphene did not persist for so long a period as D.M.B.A. Whether the elimination of the "acid" component before the disappearance of the

" alkaline " component (Fig. 4) is of significance is not known. Quite definite amounts, however, are present 24–48 hours after application and thus we have another example of a " binding hydrocarbon " in addition to those already observed.

Any interpretation of the results in Series 1 must be somewhat conjectural. It is possible that the benzpyrene component from the alkaline extract is a measure of the combination of the hydrocarbon with damaged tissue. This would account for the high value after the nitrogen mustard treatment and possibly for that after the massive dimethyl-benzanthracene treatment. On the other hand it may be that the enhanced metabolism which accompanies the hyperplasia induced by nitrogen mustard is associated with increased hydrocarbon oxidation and consequent capacity for protein-hydrocarbon combination. On this view the hyperplasia induced by a carcinogen would involve additional complex formation but this would not necessarily be involved in whatever constitutes carcinogenesis. The low value after maleic anhydride treatment might be interpreted as resulting from the reduced activity of tissue enzymes, for this substance is believed to combine with the SH groups of proteins.

Treatment with urethane, described as an effective initiating agent, does not affect the binding properties of the cell proteins. The results obtained in this investigation with benztetraphene oppose the hypothesis that skin cancer is the direct outcome of simple interaction of hydrocarbon and cell protein.

SUMMARY.

1. The amount of benzpyrene bound to epidermal protein of mouse skin has been determined after application of that carcinogen preceded by treatment with various agents which induce epidermal changes but which are not in themselves carcinogenic.

2. Bound 9 : 10 dimethylbenzanthracene has been shown to be present for 10 days after a single massive application of this hydrocarbon.

3. Benztetraphene though non-carcinogenic becomes bound to epidermal constituents in amounts comparable to benzpyrene.

I wish to thank Professor J. W. Orr for advice and information and Professor H. N. Green for the sample of benztetraphene.

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THE INTRACELLULAR METABOLISM OF 3 : 4 BENZPYRENE STUDIES OF RAT LIVER SUBJECTED TO REPEATED DOSES OF BENZPYRENE

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PREVIOUSLY Calcutt and Payne (1954*a*, 1954*b*, 1954*c*) have described the determination of the intracellular sites of metabolism of 3 : 4-benzpyrene in mouse and rat liver. These earlier experiments were limited to consideration of events within the first 24 hours after application of the hydrocarbon. As an extension of this work we have now examined the course of events in rat liver over longer time intervals. The primary purpose of the investigation was to discover whether any changes occurred in the overall distribution of metabolic sites and to determine whether metabolism was a continuous process or dependent upon the presence and integrity of any specific substrate.

MATERIALS AND METHODS.

The animals used throughout the experiments described below were rats, aged 6–9 months, of the Wistar strain. The benzpyrene was applied as a colloidal suspension in distilled water at a concentration of approximately 1 mg. per c.c. In all experiments the animals were killed five hours after being treated with benzpyrene. The livers were homogenised and fractionated into nuclei, mitochondria, microsomes and top, middle and bottom subfractions of the supernatant. The fractionation procedures and subsequent examinations for the presence of benzpyrene metabolites were carried out as in the earlier work (Calcutt and Payne, 1954*a*, 1954*b*, 1954*c*.).

The terminology devised by Weigert and Mottram (1946) is used in referring to the metabolic derivatives of benzpyrene. This is as below :

BpX_1 = 8(OR_1)—9(OH)—8, 9-dihydro-3 : 4-benzpyrene
BpX_2 = 8(OR_1)—9(OR_2)—8, 9-dihydro-3 : 4-benzpyrene
BpF_1 = 8(OR_1)—3 : 4-benzpyrene
where R_1 and R_2 are unknown radicals.

RESULTS.

The experiments comprising the work reported in this paper fall into groups and are considered as such below. During the course of the experiments several subsidiary points arose and findings in connection with these are given later.

First experiment.

A batch of male rats was injected weekly with an intraperitoneal dose of 2 mg. of benzpyrene. Two rats were taken at weekly intervals and killed. The livers

were fractionated and the presence of benzpyrene metabolites in the different fractions determined. The detailed findings are shown diagrammatically in Fig. 1. From this it will be seen that there is a change in the distribution of sites at which BpX_1 and BpX_2 are formed as the treatment is continued.

Second experiment.

This was a repetition of the previous experiment but using female animals. The results are shown in Fig. 2. Here the changes were more noticeable, metabolism having ceased or fallen below detectable limits by the time seven doses had been given.

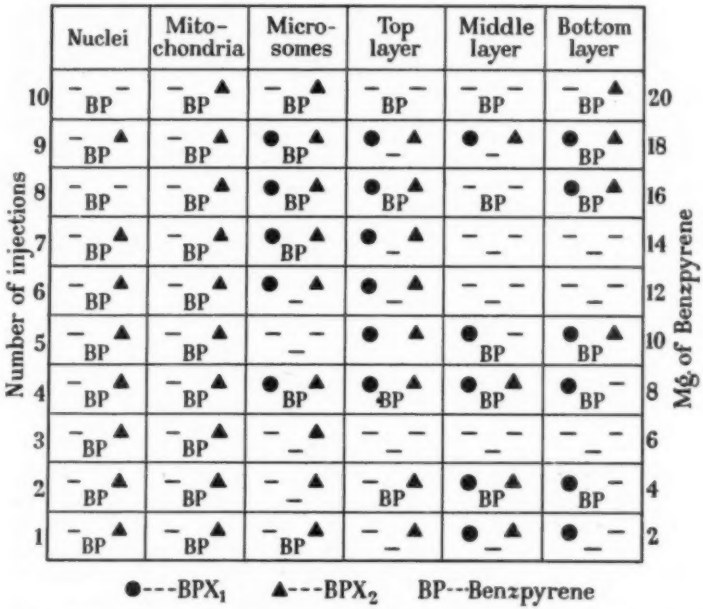

FIG. 1.—The distribution of benzpyrene metabolites in liver fractions from male rats. Each determination is based on the pooled livers from two rats. Failure to detect benzpyrene or its derivatives is indicated by a dash.

Third experiment.

Female rats of the Wistar strain are much smaller than the males, so the loading of benzpyrene per unit liver mass must have been greater in the second experiment than in the first. So a further limited series of male rats was investigated to see if metabolism of the hydrocarbon could be arrested by giving a heavier dosage of benzpyrene. This time the animals were injected twice weekly, 2 mg. per animal being given on each occasion. Findings in respect of metabolism occurring after several weeks of this treatment are shown in Fig. 3. Here again it will be seen that metabolism completely ceased or fell below detectable limits.

On various occasions during the above experiments BpF_1 and its breakdown product 8·OH benzpyrene have been found. These findings have been omitted from the figures as both these products were shown by Weigert and Mottram (1946) to be derived from the primary metabolites BpX_1 and BpX_2.

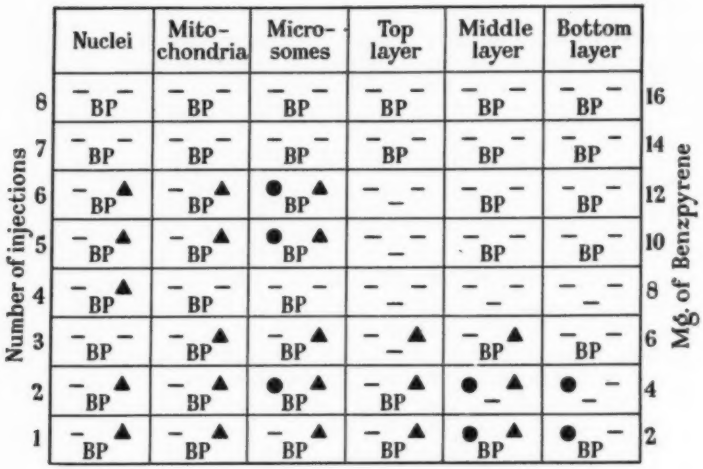

FIG. 2.—The distribution of benzpyrene metabolites in liver fractions from female rats. Symbols as in Fig. 1.

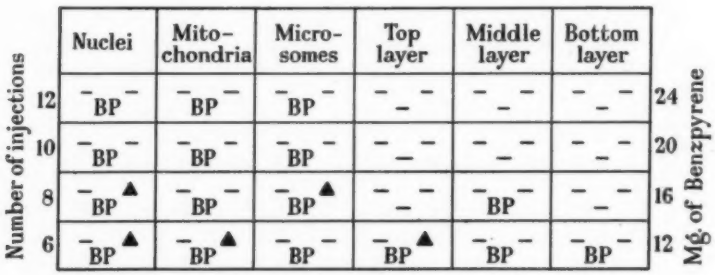

FIG. 3.—The distribution of benzpyrene metabolites in liver fractions from male rats (Experiment 3). Symbols as in Fig. 1.

Alternative metabolic pathways.

With the reduction or cessation of the formation of BpX_1 and BpX_2 it might be expected that metabolism is diverted into alternative pathways. The extracts of the various fractions. however, have given no indications of the presence of any other benzpyrene derivatives. Equally, the animals themselves have shown no signs of any increased retention of the unchanged hydrocarbon as the experi-

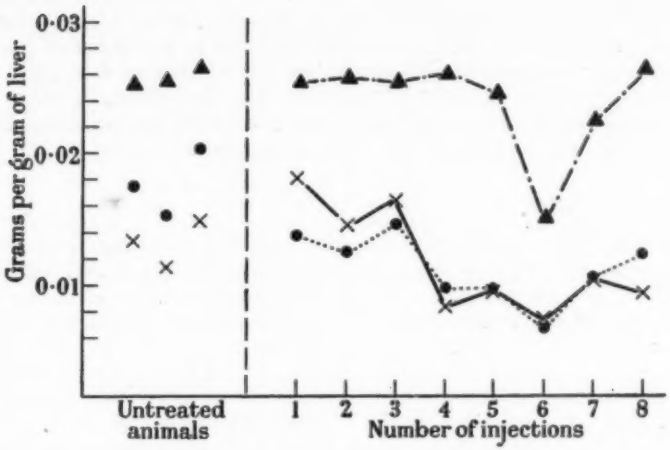

FIG. 4.—Protein concentrations of subfractions of the supernatant from female rat livers. Each determination is based on the pooled livers from two rats. Of the 2 rats receiving 6 injections the liver of 1 was grossly necrotic.

× —— Top layer. ● - - - - Middle layer. ▲ — · — Bottom Layer.

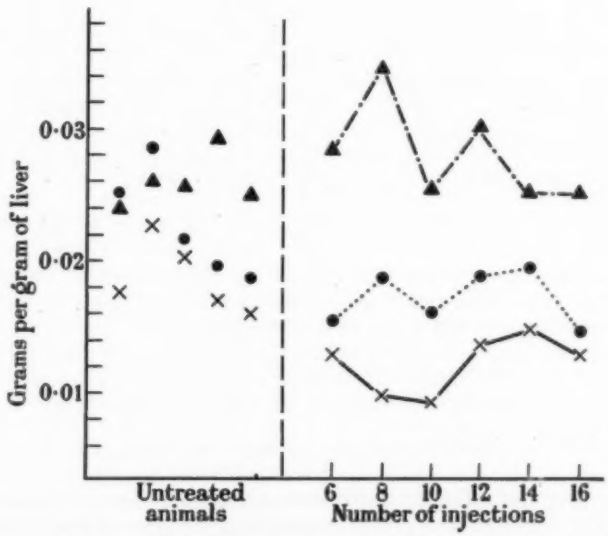

FIG. 5.—Protein concentrations of subfractions of the supernatant from male rat livers. Symbols as in Fig. 4.

ments have progressed. A possibility is that after metabolism is reduced the hydrocarbon is excreted unchanged. This is supported by the observation made in this laboratory that rats which have been subjected to a number of injections of benzpyrene show a faecal excretion of very little phenolic derivative together with a relatively large amount of unchanged hydrocarbon. Freshly injected animals, on the other hand, show a preponderance of the phenolic derivatives accompanied by only small amounts of the parent benzpyrene.

Soluble proteins of liver.

During the first experimental series we were led to suspect a change in the overall supernatant fraction from treated animals as compared with controls. So in the two subsequent series the protein concentrations of the subfractions were determined from measurements of their respective refractive indices. From these the amounts of protein per unit mass of liver used were calculated. The results for female animals are shown in Fig. 4 and the corresponding data for males in Fig. 5. The results give an impression that there is a reduction in the amounts of protein comprised in the top and middle subfractions. This point, however, requires further corroboration, particularly since the overall architecture of the liver alters with continued application of benzpyrene. After several weeks of treatment the livers show an appreciable degree of round cell infiltration and signs of degeneration, which means that the livers early in the experiment are not strictly comparable to those used later.

Lipoprotein fractions.

After the high speed centrifugation required for separation of the supernatant into subfractions. tubes containing material from untreated animals are found to have a narrow surface layer of milky material. As this fraction was noticed to be absent in some experimental runs a record was maintained of its presence or otherwise. The appearance and behaviour of this fraction suggested its possible lipoprotein nature. Some of the material was extracted from a batch of untreated rats and then purified by further centrifugation after admixture with 1·3 per cent sodium chloride solution. Tests gave the following results.

The presence of unsaturated fatty acids was shown by the blackening of osmic acid.

An ether extract gave a white deposit when diluted with twice its volume of acetone. This indicates lecithin, which was confirmed by taking up the deposit in water and obtaining typical myelin figures.

The acetone-ether fraction remaining from the above experiment gave a positive Lieberman-Burchard test for cholesterol.

A Rosenheim bismuth test for free choline was negative.

It was concluded that this fraction must be essentially the lipoproteins from the liver cells.

From the protocols of the experiments a table has been drawn showing our findings in respect of this fraction. This appears below as Table I. Although no quantitative measurements have been attempted it will be seen that this fraction either disappears completely or is much reduced.

TABLE I.—*The Lipoprotein Fractions from the Supernatant Fraction of Livers of Rats Receiving Weekly Injections of Colloidal Benzpyrene.*

Female rats.		Male rats.	
Number of injections.	Appearance of lipoprotein fraction.	Number of injections.	Appearance of lipoprotein fraction.
1	As normal	6	Trace only
2	Absent	8	Absent
3	Small amount only	10	,,
4	Absent	12	Small amount only
5	,,	14	,, ,, ,,
6	Completely absent	16	Small amount present
7	Absent		
8	Completely absent		

Descriptions of fractions are those recorded in the protocols of experiments.

DISCUSSION.

The experimental results given above only represent a preliminary survey of an extensive field, nevertheless, a number of interesting points have arisen. Of these the most important is perhaps, that the action of benzpyrene on the cell involves a number of processes which may or may not be related.

The change in the sites at which the two benzpyrene derivatives BpX_1 and BpX_2 are formed suggests that there is no specificity about the factor or factors determining which derivative is produced, but that the sequence of events is conditioned by the previous history of the fraction in question. This variation in the pattern may well be the counterpart to Boyland and Wiltshire's (1953) finding that with repeated dosing of naphthalene to rats changes occurred in the relative proportions of naphthalene diol and naphthylglucuronide excreted. These particular experiments were only run over a limited period, but the results presented suggest a tailing off in overall metabolism towards the end of the investigation. Since we have found an apparent complete cessation of metabolism of benzpyrene it may be important to determine whether a similar state of affairs can be attained using the non-carcinogenic polycyclic hydrocarbons.

The reasons for the apparent cessation in the metabolism of benzpyrene are not discernible from the present experiments. It is tempting to ascribe the phenomenon to the elimination of enzyme systems concerned in the oxidation of the hydrocarbon, but this appears very unlikely as Casu *et al.* (1951) have offered convincing evidence that the metabolic oxidation of benzpyrene is completely independent of enzyme systems. This leaves two alternative explanations. The first, that the benzpyrene ceases to penetrate the cell after a time, is disposed of by the finding of the unchanged hydrocarbon in the various fractions at a time when metabolism no longer occurs. The second, that some unidentified grouping in the cell fractions is eliminated, cannot yet be checked. It is hoped that further evidence may be obtained from a more detailed examination of the residual cell fractions which is now being undertaken.

The possible changes in amounts of soluble proteins in the livers of treated animals resembles findings with other carcinogenic agents. Using 2-acetylaminofluorene Laird and Miller (1953) found a fall in the protein content of the supernatant from liver four weeks after commencement of treatment. Later a

return to a more normal level occurred. Studies by Price, Miller, Miller and Weber (1950) are suggestive of a small decrease in the protein content of the supernatant fraction from rat livers treated with azo dyes. The supernatant fraction from hepatic tumours induced by azo dyes has been shown by Sorof and Cohen (1951) to possess a decidedly lower complement of the lower molecular weight proteins than that from normal liver. In the present experiments it is also the lower molecular weight proteins—i.e. the slower sedimenting fractions—which show signs of change.

The reduction in the lipoprotein fraction associated with the supernatant is also interesting, in that lecithin which forms part of this fraction contains choline. The absence of choline has been reported by Copeland and Salmon (1946) to result in liver tumours, whilst its presence protects against fatty infiltration and cirrhosis of the liver. Both of these conditions are known to be intimately involved with carcinogenesis of the liver. Whilst benzpyrene is not recognised as causing such changes the closely related hydrocarbon 1 : 2 : 5 : 6-dibenzanthracene was shown by Claude (1937) and Burrows and Boyland (1938) to cause cirrhosis in rabbit liver.

The evidence which has become available from this present preliminary investigation suggests a number of points for further examination. Obviously the question of the actual substrates involved in the metabolic processes must be pursued further. Equally the fact that benzpyrene induces changes in liver components similar to those produced by other carcinogenic agents demands further experiments. In this connection it must be remembered that benzpyrene has never been found to induce liver tumours, although the evidence summarised by Berman (1951, p. 119) shows that related polycyclic hydrocarbons can act in this fashion. Experiments designed to test the carcinogenicity of benzpyrene for liver are in progress and solution of this problem will aid in evaluating the possible relationship of some of the effects produced by the hydrocarbon to the carcinogenic process itself.

SUMMARY.

1. Wistar strain rats have been injected intraperitoneally at weekly intervals with colloidal benzpyrene.

2. Determinations of benzpyrene metabolites in the various intracellular fractions at weekly intervals have shown that variations occur in the sites of formation of the two derivatives BpX_1 and BpX_2. Finally all metabolism ceases or falls below detectable limits.

3. Indications were found of a fall in the amounts of the lower molecular weight proteins of the supernatant fraction.

4. Lipoproteins associated with the supernatant fractions were found to be much reduced or to disappear.

5. The experimental results and their significance are briefly discussed.

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THE INFLUENCE OF A HYPERVITAMINOSIS ON THE EFFECT OF 20-METHYLCHOLANTHRENE ON MOUSE PROSTATE GLANDS GROWN *IN VITRO*.

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EXPERIMENTS suggest that vitamin A influences the direction and type of differentiation in the epidermis and mucous epithelium. Thus lack of the vitamin was found to produce squamous keratinising epithelium in organs which normally are lined by mucous producing ciliating cells, as in the respiratory tract of rats and guinea-pigs (Wolbach and Howe, 1925, 1928) while in vitamin A deficient rats the prostate glands showed squamous metaplasia of the alveolar epithelium (Bern, 1952).

On the other hand, high doses of the vitamin given either systemically (Studer and Frey, 1949) or locally (Sabella, Bern and Kahn, 1951) caused a thickening of the epidermis in rats, particularly of the stratum granulosum. Recently, Fell and Mellanby (1953) grew embryonic chick ectoderm in the presence of excess vitamin A *in vitro* and found that keratinisation was suppressed and mucous-producing ciliating epithelium, histologically similar to that of the nasal mucosa, differentiated instead.

It has been shown in previous work (Lasnitzki, 1951) that 20-methylcholanthrene added to the medium of mouse prostate glands *in vitro* induces hyperplasia and squamous metaplasia of the alveolar epithelium, changes which become more pronounced after withdrawal of the carcinogen. It seemed possible that high doses of vitamin A might alter the response of the tissue to 20-methylcholanthrene and in the present investigation the influence of excess vitamin A on the carcinogenic effect was studied.

Three groups of experiments were made. In the first, vitamin A alone was added to the medium, in the second excess vitamin was given simultaneously with 20-methylcholanthrene, and in the third it was administered to cultures previously treated with the carcinogen. Two concentrations of vitamin and of 20-methylcholanthrene were used.

MATERIAL AND TECHNIQUE.

The explants consisted of the ventral prostate glands of C3H mice of 3 months of age. They were grown by the watchglass technique (Lasnitzki, 1951, 1954) on clots consisting of a mixture of fowl plasma, rat plasma, chick embryo extract and human serum containing the carcinogen. The total amount of medium per watchglass was 0·5 ml.

One half of the paired gland was grown with excess vitamin A, alone or with the carcinogen added either simultaneously or subsequently, while the other half

* Sir Halley Stewart Fellow.

was kept as a control in normal medium or with 20-methylcholanthrene alone (Me control). The period of cultivation was either 10 or 20 days, and the explants were transferred to a fresh medium every 3–4 days. In all, 102 explants, corresponding to 51 whole ventral prostate glands, were grown.

The fowl plasma used for the experimental cultures contained either 2000 or 3000 i.u. per 100 ml. of added vitamin A in alcoholic solution. Its strength was such that the final plasma contained 0·2 per cent or less ethanol. In each experiment the same quantity of ethanol was added to the control plasma. Most of the vitamin in the artificially reinforced plasma was in a fat soluble form in contrast to the natural vitamin which is water soluble (Fell and Mellanby, 1952). Since the fowl plasma constituted one half of the total medium the final concentrations amounted to 1000 or 1500 i.u. per 100 ml. The vitamin A content of normal fowl plasma is 200–300 i.u./100 ml. while that of normal mouse plasma is 20–60 i.u./100 ml. The concentration in the normal control medium was therefore slightly hypervitaminotic for the mouse prostate gland.

The carcinogen was suspended in human serum ; 0·06 ml. or 0·15 ml. of a 0·3 per cent solution of 20-methylcholanthrene in acetone was shaken into 4 ml. of serum. 0·025 ml. or one drop of this suspension containing 1 μg. or 2·5 μg. were added to the culture medium bringing the final concentrations to 2μg. or 5 μg./ml.

The experiments were arranged as follows :

I. *Vitamin A alone.*

Experimental cultures were grown with (1) 1000 i.u. and (2) 1500 i.u. of vitamin A for 11 days ; the two sets of controls were kept in normal medium for the same period.

II. *20-methylcholanthrene combined with vitamin A.*

(*a*) Control cultures were kept in medium containing 2 μg. of 20-methylcholanthrene alone for 10 days and then fixed (Me controls).

(*b*) Experimental cultures were treated with 2 μg. of 20-methylcholanthrene and 1000 i.u. of vitamin A for the same period and then fixed.

(*c*) Control cultures (Me controls) were kept in medium containing (1) 2 μg. and (2) 5 μg. of 20-methylcholanthrene per ml. for 11 days followed by cultivation in normal medium for 9 days.

(*d*) Experimental cultures were kept in medium containing (1) 2 μg of 20-methylcholanthrene and 1000 i.u. of vitamin A and (2) 5 μg of 20-methylcholanthrene and 1500 i.u. of vitamin A for 11 days followed by cultivation in normal medium for 9 days.

III. *20-methylcholanthrene followed by vitamin A.*

(*a*) Control cultures (Me controls) received (1) 2 μg. and (2) 5 μg. of 20-methylcholanthrene for 11 days followed by cultivation in normal medium for 9 days.

(*b*) Experimental cultures were grown for 11 days with (1) 2 μg. and (2) 5 μg. of 20-methylcholanthrene followed by cultivation in media containing (1) 1000 i.u., and (2) 1500 i.u. of vitamin A for 9 days.

At the end of the experimental period the explants were fixed in 2 per cent acetic Zenker's fluid for 30 minutes, washed in distilled water, dehydrated,

embedded in paraffin and serial sections of 6 μ thickness were cut. The sections were stained with haematoxylin-eosin, or by the periodic-acid Schiff method with diastase digestion.

The symbols + and ++ are used in Tables I, II and III to give a measure of the incidence and extent of hyperplasia and metaplasia in treated cultures. Hyperplasia + signifies that up to half the total number of alveoli in the explant show epithelial proliferation to form 3–6 layers of cells; hyperplasia ++ signifies that half or more of the total number of alveoli are hyperplastic with partial or complete occlusion of the lumen in half the hyperplastic alveoli. Metaplasia + means squamous changes in up to half the number of hyperplastic alveoli, ++ in half to all hyperplastic alveoli.

RESULTS.

The ventral prostate gland of a 3-month-old mouse consists of alveoli lined with one row of cuboidal or cylindrical epithelium (Fig. 1) which is rarely folded, in contrast to that from younger animals. The lumina are fairly wide and the secretory epithelium often shows pycnosis and is shed into the lumen.

Control cultures.—Two types of growth can be distinguished *in vitro*. The proliferation of unorganised fibroblasts which surround the explant and are removed at every transfer and the development of differentiated alveoli which are carried over at subcultivation (Fig. 2). The newly formed alveoli resemble those *in vivo*, are lined with one layer of cuboidal or cylindrical secretory epithelium the free surface of which is usually covered by a thin homogeneous layer of PAS positive material. Occasionally reserve cells are seen between the lining epithelium and the basement membrane. The epithelium *in vitro* is more folded and the lumen is narrower than *in vivo* and degeneration is absent in the layer bordering the

EXPLANATION OF PLATE.

FIG. 1.—Ventral prostate gland in 3-month-old mouse *in vivo*. Haematoxylin-eosin. × 115.

FIG. 2.—Ventral prostate gland from 3-month-old mouse grown in normal medium for 3 weeks. Haematoxylin-eosin. × 115.

FIG. 3.—Similar explant grown for 11 days with 5 μg. of 20-methylcholanthrene and maintained in normal medium for 9 days. Note hyperplasia and squamous metaplasia of alveoli. Haematoxylin-eosin. × 115.

FIG. 4.—Hyperplastic alveolus from similarly treated explant at higher magnification. Note degeneration and sloughing of luminal epithelium. × 350.

FIG. 5.—Ventral prostate gland from 3-month-old mouse grown with 5 μg. of 20-methylcholanthrene and 1500 i.u. of vitamin A for 11 days and maintained in normal medium for 9 days. Note scarcity of squamous changes and preponderance of columnar epithelium. Haematoxylin-eosin. × 120.

FIG. 6 and 7.—Hyperplastic alveoli from similarly treated explants showing (6) columnar epithelium, and (7) stratification with columnar and hexagonal cells and preservation of the secretory epithelium. Haematoxylin-eosin. × 560.

FIG. 8 and 9.—Stratified alveoli in Me + A treated explants showing mitosis below the luminal epithelium (8), and among columnar cells (9). In (9) note bridges between cells and between luminal epithelium and cells underneath. Haematoxylin-eosin. × 1300.

FIG. 10.—Similar explant treated for 11 days with 5 μg. of 20-methylcholanthrene and continued for 9 days with 1500 i.u. of vitamin A. Note scarcity of hyperplasia. Haematoxylin-eosin. × 120.

FIG. 11.—Alveolus from similarly treated explant showing mild hyperplasia. All the cells are of the basal or reserve cell type. Haematoxylin-eosin. × 350.

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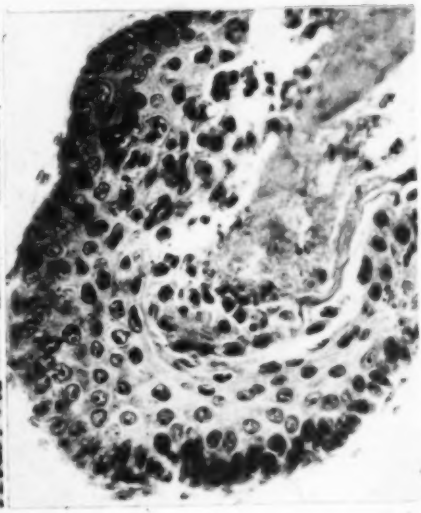

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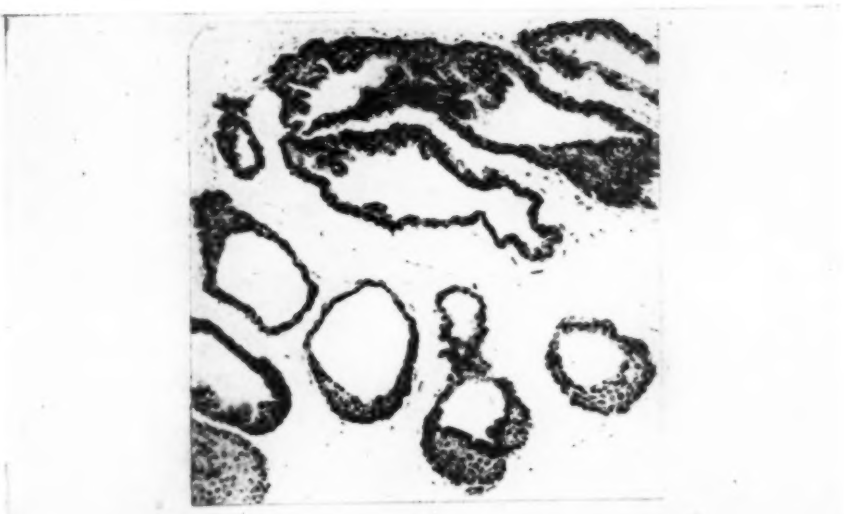

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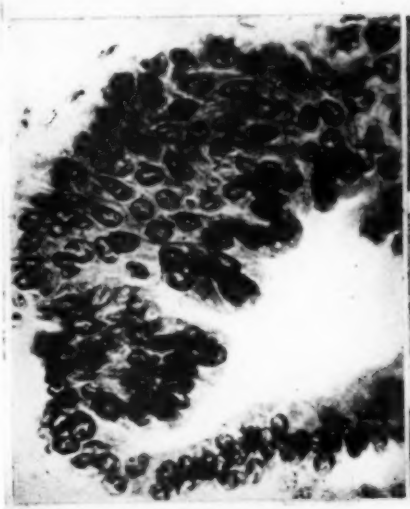

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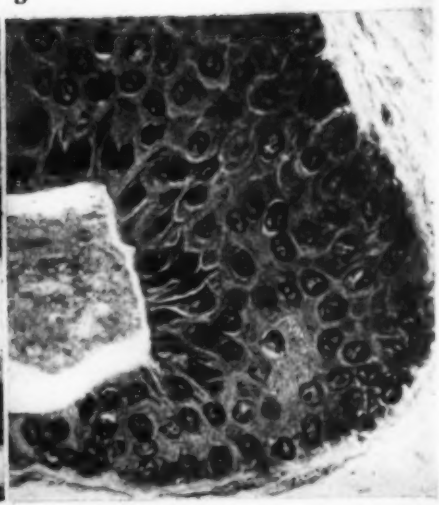

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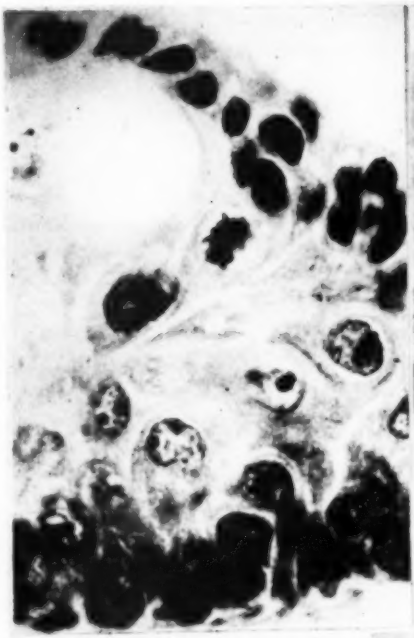

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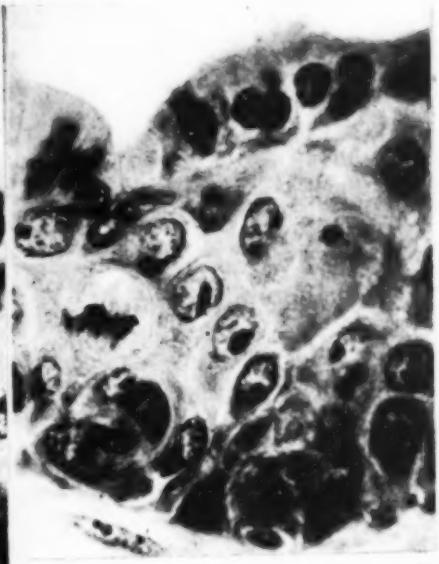

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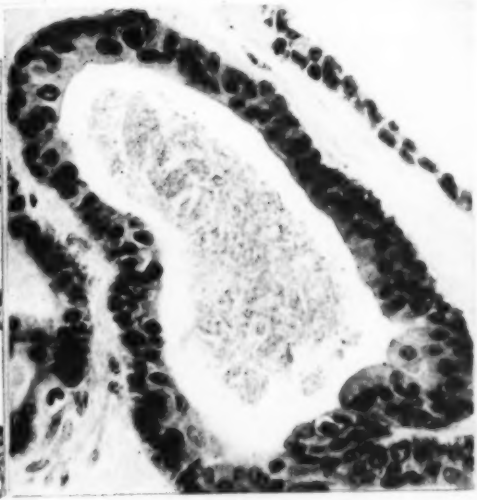

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lumen. The connective tissue is slightly increased as compared with the gland *in vivo* and alveoli are surrounded by concentric strands of collagenous fibres, while fibroblasts and thinner fibres diffusely fill the interalveolar spaces.

I. *Vitamin A alone.*

Explants grown for 10 days with 1000 i.u. of vitamin A alone show no changes as compared with their untreated controls. The folding and height of the alveolar epithelium and the amount of connective tissue are similar in both sets of cultures. In explants treated with the higher dose (1500 i.u.), however, more PAS positive material can be distinguished, which is deposited in coarse granules between and in front of the secretory cells.

II. *20-methylcholanthrene combined with Vitamin A.*

The effect on prostatic epithelium of 20-methylcholanthrene alone has been described in detail elsewhere (Lasnitzki, 1951), so need be only briefly recapitulated here and those points emphasized which are of importance in connection with the action of vitamin A.

Since 1951 many prostate cultures from mice of different ages have been treated with the carcinogen and the results obtained confirmed those of the earlier experiments. Application of the carcinogen is followed by increased proliferation of the reserve cells resulting in many layers of densely crowded cells, which partially or completely occlude the alveolar lumen. At the beginning of treatment these cells are round or columnar with distinct spherical or oval basophilic nuclei. Between the 10th and 20th day of growth, i.e. after withdrawal of the carcinogen which is discontinued after the 10th or 11th day, hyperplasia becomes more extensive and stratification and squame formation of the hyperplastic epithelium begins (Fig. 3). In hyperplastic alveoli the periphery is occupied by actively dividing cells corresponding to the basal cells in stratified epithelium, and an inner layer of prickle cells. There is a gradual increase in cell size towards the centre due to enlargement of the cytoplasm which stains pink with eosin. In such cells the nuclei are usually shrunk and stain very faintly with haemotoxylin-eosin. The original secretory epithelium degenerates, is shed and replaced by flat cells orientated with their long axis parallel to the lumen. In some cultures keratin is formed from such cells, in others they degerate without cornifying and are sloughed (Fig. 4). The onset of squamous metaplasia and the proportion of basal to

TABLE I.—*Comparison of the Effect of 20-methylcholanthrene Alone and of 20-methylcholanthrene and Excess Vitamin A combined for 10 days.*

Treatment.		2 μg. Me + 1000 i.u. A	
		Hyperplasia.	Squamous metaplasia.
Me 10 days	{	1++ (6)*	..
		3+ ..	6—
		2— ..	..
Me + A 10 days	{	4+ (7)	7—
		3— ..	..

* Figure in brackets gives number of treated explants.

prickle and cornifying cells is related to the concentration of the carcinogen: after a lower dose there are usually more basal cells and squamous changes occur later, whereas after the higher dose more squamous cells are formed and the onset of metaplasia is accelerated.

Of six explants treated for 10 days with 20-methylcholanthrene three show slight and one more extensive hyperplasia, while squamous changes have not yet begun (Table I, Column 1).

Addition of 1000 i.u. per 100 ml. of vitamin A to the medium does not modify these effects of the carcinogen. Thus, in 4 out of 7 treated explants there is mild hyperplasia but no squamous metaplasia (Table I, Column 2).

Explants grown with 2 μg. of 20-methylcholanthrene for 11 days and maintained in normal medium for a further 9 days, show hyperplasia which is extensive in 3 and less so in 4 cultures, while marked metaplasia appears in 4 explants. After 5 μg., both hyperplasia and squamous metaplasia are more pronounced than after the lower concentration (Table II, Column 1).

TABLE II.—*Comparison of the Effect of* (1) *20-methylcholanthrene Alone and of 20-methylcholanthrene Combined with Excess Vitamin A for* 11 *days, followed by Cultivation in* (2) *Normal Medium and* (3) *maintained with Excess Vitamin A followed by Normal Medium.*

Treatment.	2 μg. Me. Hyperplasia.	2 μg. Me. Squamous metaplasia.	5 μg. Me. Hyperplasia.	5 μg. Me. Squamous metaplasia.
(1) Me 11 days normal medium 9 days	3++ (7) 4+ ..	4++ 2+ 1−	5++ (6) 1+ ..	5++ 1+ ..
	2 μg. Me + 1000 i.u. A		5 μg. Me + 1500 i.u. A.	
(2) Me + A 11 days normal medium 9 days	2++ (6) 4+	2+ 4−	4++ (6) 2+	2+ 4−
(3) Me + A 11 days, A 3 days, normal medium 6 days	4+ (6) 2− ..	6−	1++ (6) 4+ 1−	6− .. ..

Application of 1000 and 1500 i.u. of the vitamin simultaneously with 20-methylcholanthrene for 11 days followed by cultivation in normal medium for 9 days does not alter the incidence or extent of epithelial hyperplasia (Table II, Column 2), but the hyperplastic epithelium presents significantly different features from those of the (Me) controls (Fig. 5). At both concentrations only 2 out of 6 glands show mild squame formation which, moreover, is confined to a very small number of alveoli. In other hyperplastic alveoli the epithelium consists either of heaped up small round or oval cells, which towards the centre sometimes, but not always, assume columnar shape (Fig. 6), or stratification takes place in such a way that the small peripheral cells are associated with layers first of columnar and then hexagonal cells; both types show intercellular cytoplasmic bridges (Fig. 7). In contrast to the prickle cells in carcinogen-treated cultures, the cytoplasm and nuclei of these hexagonal cells are basophilic. At the lumen they are joined by cytoplasmic bridges with the innermost layer of cylindrical secretory epithelium. In Me controls the secretory epithelium usually degenerates, is sloughed and replaced by a layer of flat cells. After 2 μg. of 20-methylcholan-

threne keratin is formed at the lumen ; after 5 μg., no keratin appears and instead the innermost layer of flat cells degenerates (Fig. 4). Addition of the vitamin completely suppresses the keratin formation or degeneration of the innermost layer, while the secretory epithelium is preserved in most of them. Cell divisions can be observed in all cell layers including the secretory epithelium in contrast to Me controls in which mitosis is confined to the basal cells (Fig. 8 and 9).

In glands treated with the vitamin for 3 days after withdrawal of the carcinogen followed by 6 days' cultivation in normal medium the differentiation of the hyperplastic epithelium is similarly altered. In addition, squame formation is entirely absent and the incidence of hyperplasia reduced (Table II, Column 3). Slides stained with PAS show increased mucin production in unchanged alveoli and in those with early hyperplasia, but in alveoli showing marked hyperplasia only faint traces of mucin can be distinguished in the secretory lining.

III. *20-methylcholanthrene followed by vitamin A.*

Application of the vitamin for a further 9 days to explants treated for 11 days with the carcinogen inhibits or suppresses squamous changes and significantly decreases the incidence of hyperplasia (Table III, Column 2). Thus, metaplasia is missing in all, and hyperplasia found in only 2 of 6 cultures treated with 2 μg. of 20-methylcholanthrene followed by 1000 i.u. of vitamin A ; after the higher concentration 2 out of 7 cultures show slight squamous changes and 6 mild hyperplasia (Fig. 10). Stratification with squamous changes is confined to very few alveoli ; elsewhere it is absent and the hyperplastic epithelium consists of a few rows of small round or columnar cells (Fig. 11).

TABLE III.—*The Effect of Excess Vitamin A on Explants Treated Previously with 20-methylcholanthrene.*

Treatment.	2 μg. Me. Hyperplasia.	2 μg. Me. Squamous metaplasia.	5 μg. Me. Hyperplasia.	5 μg. Me. Squamous metaplasia.
(1) Me 11 days normal medium 9 days	2++ (6)	4+	9++ (10)	9++
	2+	2−	1+	1−
	2−	..	..	..
	2 μg. Me + 1000 i.u. A.		5 μg. Me + 1500 i.u. A.	
(2) Me 11 days A 9 days	2+ (6)	6−	6+ (7)	2+
	4−	..	1−	5−

DISCUSSION.

Excess vitamin A added alone to the medium of mouse prostate glands does not influence the normal development of the epithelial structures although the higher concentration causes a slight increase in the production of mucin. Simultaneous addition of the vitamin with the carcinogen, however, reduces squamous changes, suppresses keratin formation and prevents the degeneration of the secretory lining epithelium.

This result again indicates that the vitamin is an important factor in the control of both keratin- and mucin-formation. In recent experiments on spayed rats Kahn (1954) showed that vitamin A applied locally prevented cornification of the vagina after oestrogen treatment and produced stratified cuboidal epithelium

instead. Dziewiatowsky (1954) provided direct experimental proof that the vitamin promotes mucin production; thus the incorporation of S^{35} as sulphate sulphur into mucopolisaccharides was increased in A deficient rats treated with vitamin A. Similarly, Fell, Mellanby and Pelc (1954) found that the mucous metaplasia of embryonic chick ectoderm grown *in vitro* with excess vitamin A was associated with a marked uptake of S^{35} sulphate.

It is probable that the action of the vitamin is exerted primarily on the reserve or basal cells which at this stage may be capable of both types of differentiation: squamous or columnar. An interesting feature is the appearance in stratified alveoli of cytoplasmic bridges between columnar and hexagonal cells, but particularly those joining the luminal epithelium with the cell layer underneath. Cell bridges do not normally occur in columnar epithelium. The hexagonal cells resemble the prickle cells of the epidermis save for their basophily, and may be considered either not fully differentiated precursors of the eosinophilic prickle cells or a variation of the columnar cell type.

The presence of cytoplasmic bridges between the luminal epithelium and the layer of hexagonal cells suggests that the secretory elements are derived by differentiation from the former. On the other hand, since cell divisions are observed among the luminal cells it is possible that they grow independently and in spite of the stratification going on beneath them.

Administration of the vitamin to explants treated previously with 20-methylcholanthrene not only suppresses squamous changes but reduces the increased cell multiplication due to the carcinogen. The greater effectiveness of the vitamin in this group of experiments may be due to its increased uptake in the absence of competition from the carcinogen.

The reduction in hyperplasia is difficult to interpret since the mechanism by which it is brought about is, as yet, unknown. Two facts are clear however. It is unlikely to be due to a direct inhibition of mitosis since the author has shown (Lasnitzki, 1955) that excess vitamin A added to the medium of chick fibroblasts *in vitro* increases the mitotic rate as well as cell migration. Further, it is not affected by destruction of the hyperplastic epithelium like that which follows oestrone treatment of mouse prostate glands *in vitro* pretreated with 20-methylcholanthrene (Lasnitzki, 1954). It seems probable that the effect is an indirect one and that the vitamin affects the mechanism controlling the ratio of cell multiplication and differentiation. It may thus restore the normal balance of the two processes by reversing the disturbance due to the carcinogen. How this is brought about is not known.

The results suggest that the vitamin A level may have some influence on the rate of development of epithelial tumours. A slight increase above the normal may accelerate tumour growth by suppressing squamous changes, while higher concentrations, by antagonising the action of the carcinogen, may retard it.

SUMMARY.

Ventral prostate glands from 3-month-old C3H mice were grown *in vitro* by the watchglass technique with (1) excess vitamin A alone and (2) with a combination of 20-methylcholanthrene and excess vitamin applied simultaneously and successively. Two concentrations of carcinogen and vitamin were studied.

The addition of the vitamin alone does not influence growth and development

of the prostatic epithelium, but the higher concentration slightly increases mucin production.

Addition of 20-methylcholanthrene alone induces hyperplasia and squamous metaplasia of the alveolar epithelium.

Simultaneous addition of vitamin A and 20-methylcholanthrene followed by cultivation in normal medium does not alter the incidence and extent of epithelial hyperplasia but suppresses keratin formation and prevents degeneration of the secretory lining epithelium. The hyperplastic epithelium consists of small basal cells, columnar elements and hexagonal basophilic cells with intercellular bridges. The latter are considered either not fully differentiated precursors of eosinophilic prickle cells or atypical columnar epithelium.

Addition of excess vitamin A to glands previously treated with 20-methylcholanthrene likewise prevents keratinisation, but it also decreases significantly the incidence and degree of hyperplasia.

I am greatly indebted to the late Sir E. Mellanby, F.R.S. for the provision of the plasma used in these experiments. I should also like to thank Dr. Honor B. Fell, F.R.S. for constructive criticism in the preparation of the manuscript, Mr. R. J. C. Stewart, chief technician at the Nutrition Building, National Institute for Medical Research, for preparing the blood plasma, and Mr. G. C. Lenney, Strangeways Research Laboratory, for the microphotographs.

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POLYCYCLIC HYDROCARBONS IN CIGARETTE SMOKE: THE CONTRIBUTION MADE BY THE PAPER.

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IN previous studies on cigarette smoke it has been shown that a number of polycyclic hydrocarbons are present (Commins, Cooper and Lindsey, 1954; Cooper, Lindsey and Waller 1954; Cooper and Lindsey, 1955). The occurrence of such substances in the smoke from cigarette paper has also been announced independently in England (Cooper and Lindsey, 1954) and America (Lefemine, Grand and Ayre, 1954; Rand, 1954, private communication). A full account of this and related work follows.

Preparation of Smoke from Cigarette Paper.

It was decided to prepare the smoke in a manner resembling the normal combustion of a cigarette as closely as possible. A number of "cigarettes" were prepared for us entirely from cigarette paper. They were filled with paper shredded to the same texture as the tobacco normally used, and were of approximately the same dimensions as the cigarettes commonly sold in this country, namely, 0·7 cm. in diameter and 7·0 cm. long. Their average mass was 0·356 g., and hence they were much lighter than tobacco-filled cigarettes, which weigh about 1 g. each. They were smoked five at a time in the machine previously described (Cooper and Lindsey, 1955) under conditions so adjusted that the rate of combustion (mass/unit time) was the same as with tobacco-filled cigarettes. The stubs were discarded at a length of 1·5 cm., so that, as in normal smoking, about 0·8 of the cigarette was consumed.

The temperature attained during combustion were measured by inserting a thermocouple through the covering paper and allowing the combustion zone to advance over it. The highest temperature recorded within the hot zone during suction was 655° C., but it was almost impossible to measure the swing of temperature from the quiescent state to the suction state because of the rapid rate of travel of the burning zone. The temperatures measured were not so constant as when tobacco-filled cigarettes were used. Surface temperatures were measured by a disappearing-filament pyrometer and estimated as being above 900° C.

The temperatures in the hot zone of paper cigarettes are not greatly different from those in tobacco cigarettes, which remain very constant at near 650° C. in the smouldering state and rise to about 700° C. with suction. Surface temperatures of 900° C. were also observed with these cigarettes.

Analytical Techniques.

The smoke from the paper cigarettes was treated exactly as for real cigarettes (Cooper and Lindsey, 1955) and the compounds present in the neutral cyclohexane

solution detected and determined by the methods of chromatography and absorption spectrophotometry. It was found that with the smoke from paper the amount of background absorption given by the neutral cyclohexane solution was far less than in the analysis of cigarette smoke. The various compounds were therefore easier to separate and identify by chromatography. The results for the smoke obtained from 1000 " cigarettes " are shown in Table I, where the amount of paper consumed is computed from the average weight of the cigarettes and the fact that about one-fifth remains as a stub.

TABLE I.—*Polycyclic Hydrocarbons from 290 g. paper in Micrograms.*

Acenaphthylene	24·0
Anthracene	12·6
Phenanthrene	49·1
Pyrene	49·5
Fluoranthene	49·1
3 : 4-Benzpyrene	11·7
1 : 12-Benzperylene	6·5

The amount of phenanthrene was determined by the peak heights at 252 and 293 mμ. and is likely to be somewhat in error because of the high background absorption at these wavelengths.

DISCUSSION.

If it be assumed that the conditions and the products of combustion in a paper cigarette, burnt by intermittent suction, are the same as for the paper on a tobacco-filled cigarette, it may be computed that the paper covers (weighing on an average 0·0417 g. each) contribute only a fraction of the total polycyclic hydrocarbons present in the whole smoke from cigarettes. Thus Table II shows the amounts of hydrocarbons found in the smoke from 500 cigarettes (Cooper and Lindsey, 1955) and the calculated yield from the paper consumed.

TABLE II.—*Polycyclic Hydrocarbons from 500 Cigarettes in Micrograms.*

	From 440 g. cigarettes consumed.	From 16·7 g. paper. Calculated yield.
Acenaphthylene	20·5	1·41
Anthracene	48·0	0·74
Pyrene	55·0	2·92
3 : 4-Benzpyrene	4·0	0·69
1 : 12-Benzperylene	0·5	0·38

The differences between the amount of each hydrocarbon found in cigarette smoke, and the yield from 0·8 of the cigarette paper calculated from the data given in Table I, one could assume to be the amount provided by the tobacco. However, an unknown amount of smoke is trapped in the stubs and with tobacco cigarettes this may be more than with paper cigarettes. Experiments have therefore been commenced to find how much of each of the polycyclic hydrocarbons is present in the residual stubs and how much is formed when cigarette tobacco is burnt in a pipe. Preliminary investigations show that the stubs retain a considerable amount of smoke and that tobacco burned almost completely in a pipe produces more hydrocarbons than would be obtained when the same amount is burned less completely in a cigarette.

Whatever may be the proportion of the total smoke products held in the stub of a cigarette, the paper makes a contribution to the polycyclic hydrocarbons present in the main stream smoke under normal smoking conditions. As these compounds have now been proved to be formed from cellulose, it is likely that the related pectins, hemicelluloses and sugars known to be present in tobacco may also give rise to varying amounts of polycyclic hydrocarbons, and an investigation of this matter has been commenced.

It is of interest to compare the work of the American teams under Lefemine and Rand with the above results. Their cigarette paper was burnt by continuous suction, no attempt being made to approximate to the conditions of normal smoking. Although the products may be the same as were obtained from the paper "cigarettes" this fact cannot be inferred from the published results which refer only to the qualitative detection of 3 : 4-benzpyrene by spectrophotometry after chromatographic separation (Lefemine, Grand and Ayre, 1954; Rand, 1954, private communication). No mention is made by these workers of the many easily recognised related polycyclic hydrocarbons that separate in the now well-established sequence by alumina chromatography.

Selkopf (1955) in a very recent publication has demonstrated the presence of naphthalene, anthracene and traces of 3 : 4-benzpyrene in cigarette smoke obtained by artificial smoking, and has confirmed the temperatures which exist in the hot zone during smoking.

SUMMARY.

1. "Cigarettes" made entirely of paper when burnt in a smoking machine give rise to smoke containing the polycyclic hydrocarbons, anthracene, pyrene, fluoranthene, 3 : 4-benzpyrene, 1 : 12-benzperylene, acenaphthylene and phenanthrene.

2. The quantities of these hydrocarbons are far too small to account for the amounts found in the main stream smoke of a cigarette and it has been computed that the tobacco is the main source of such compounds.

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TUMOUR-PROMOTING ACTION OF CROTON OIL FRACTIONS.

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SINCE Berenblum (1941) demonstrated the power of croton oil, the expressed or extracted oil of the seeds of *Croton tiglium* (L.), to promote skin-tumour formation in the mouse, its use has come to have an important place in investigations of the process of carcinogenesis. Its well-known pharmacological properties had stimulated several attempts, some time before this, to separate the active principle or principles. Cherbuliez and his colleagues (Cherbuliez, Ehninger and Bernhard, 1932), who review work up to that date, separated a resinous substance from the oil by solvent partition between petroleum ether and 90 per cent methanol. This resin was strongly vesicant; it was concentrated in the latter solvent, while most of the purgative principle of the oil remained in the former. A further purification of the vesicant principle was achieved by other physical procedures. Berenblum (1941) subjected the oil to solvent partition between petroleum ether and 90 per cent methanol, and obtained a resin from the latter solvent which was approximately ten times more potent as a promoter of skin tumour formation than the original oil.

In the present investigation fractionation of croton oil, first by methods based on those of Cherbuliez, and then by chromatographic analysis, was undertaken. Fractions obtained at successive stages were tested for tumour-promoting power by methods which have been previously described (Gwynn and Salaman, 1953).

METHODS.

Mice.—Stock albino mice of the "S" strain (Gwynn and Salaman, 1953) were used. They were fed on a cube diet prepared according to the Rowett Institute formula (Thomson, 1930*a* and 1930*b*), and water *ad libitum*, and were 2 to 3 months old at the beginning of the experiments.

Materials.—Croton oil, obtained by expression from crushed croton seeds, was supplied by Messrs. Stafford Allen and Sons.

9 : 10 dimethyl-1 : 2 benzanthracene (D.M.B.A.) was supplied by Messrs. Light & Co.

Analytical grades of organic solvents were used throughout.

Alumina (Grade II) was supplied by Messrs. Savory & Moore.

Primary treatment.—An initial application of 0·2–0·3 ml. of 0·1 or 0·15 per cent 9 : 10 dimethyl-1 : 2 benzanthracene (D.M.B.A.) in acetone was made to the whole back, after the hair had been clipped.

Interval.—Before further treatment a period varying between 3 to 4½ weeks

was allowed to elapse. The exact length of this interval, in view of Berenblum and Shubik's (1949) findings, was not considered to be critical.

Secondary treatment.—Fractions to be tested for promoting action were dissolved in acetone, in concentrations given below, and 0·3 ml. of the solutions were applied to the back of each mouse weekly, or sometimes more frequently, for periods of 9 to 19 weeks.

Conditions within each experiment were strictly uniform, each containing its appropriate control, enabling quantitative comparisons to be made. The above-mentioned variations in concentrations of D.B.M.A. applied as primary treatment, in the interval between primary and secondary treatments, and in the duration and frequency of secondary treatment, occurred only between different experiments where qualitative, or semi-quantitative, comparison was all that was required.

EXPERIMENTAL.

I. Preparation of Cherbuliez' resin.

The method closely followed the first part of Cherbuliez' procedure.

A weighed amount of croton oil was dissolved in approximately twice its volume of petroleum ether (B.P. 60–80° C.), and shaken with one-half volume of 90 per cent methanol/aq. After separation the methanol layer was run off, and the petroleum ether solution re-extracted 2 to 5 times with 90 per cent methanol. The methanol fractions were pooled, and reduced by distillation in partial vacuum at 60° C. The last traces of methanol and water were removed in a vacuum dessicator over anhydrous $CaCl_2$. The resulting resinous material varied between 2 to 4 per cent by weight of the original oil. The petroleum ether fraction was also evaporated to dryness, and an oily residue remained.

In a typical experiment, from 20 g. croton oil 0·7 g. of the resin and 18·6 g. of the oily material were obtained, i.e. yields of 3·5 per cent and 93 per cent of the original oil respectively. These fractions were tested for promoting action by the method described above, in concentrations corresponding approximately to the amounts present in a 0·5 per cent solution of the original oil. The results are given in Table I.

TABLE I.—*Test of "Cherbuliez Resin" for Tumour-promoting Action.*

Substance	Yield (%).	Concentration in acetone applied (%).	Total tumours.	Tumour-bearing mice/survivors.	Tumours per survivor.
Original croton oil	—	0·5	66	9/15	4·4
Resin	3·5	0·01	28	5/16	1·8
Oily residue	93	0·5	28	7/14	2·0

Sixteen mice in each group. Primary treatment: 0·3 ml. o·1 per cent D.M.B.A./acetone. Interval: 5 weeks. Secondary treatment: 0·3 ml. of test solution applied to skin weekly for 11 weeks. Tumours counted at end of secondary treatment, in this and subsequent experiments.

They shew that the resin, when applied at a concentration 50 times more dilute than the original oil, produced about half the tumour incidence. It is probable, therefore, that the promoting agent has been considerably concentrated in the resin, although a substantial part of the activity remains in the oily residue.

II. *Preparation of Cherbuliez' vesicant.*

Further fractionation of the resinous substance obtained in the first experiment was undertaken, by a method similar to the second part of Cherbuliez' procedure for preparing the vesicant material described by him. The preparation of Cherbuliez resin was carried through to the stage of removal of methanol by evaporation in partial vacuum (see Experimental, Section I) leaving an aqueous suspension of the resin. This was washed with 3 successive lots of petroleum ether. The aqueous layer was then extracted with 5 lots of ethyl ether, and the pooled ethereal extracts treated with 3 lots of 0·1 N aqueous NaoH, then washed 5 times with distilled water. The ethereal layer was distilled in vacuum, and the residue taken up in 80 per cent methanol. This solution was washed with petroleum ether, freed from alcohol as before, and the resulting aqueous suspension extracted with ethyl ether. After removal of the ether by vacuum distillation a yellow solid foam remained, similar to that described by Cherbuliez.

This material was tested for tumour-promoting action by the method described above. It was strongly irritant. The maximum tolerated concentration, i.e. that giving slight scabbing, was 0·005 per cent, and this was used. The results are given in Table II.

TABLE II.—*Test of "Cherbuliez Vesicant" for Tumour-promoting Action.*

Yield as per cent of resin.	Concentration in acetone applied.	Total tumours.	Tumour-bearing mice/survivors.	Tumours per survivor.
12%	0·005%	8	4/13	0·6

Sixteen mice. Primary treatment: 0·2 ml. 0·15 per cent D.M.B.A./acetone. Interval: 3 weeks. Secondary treatment: 0·3 ml. of test solution applied to skin weekly for 19 weeks.

By comparing Tables I and II it will be seen that the tumour incidence resulting from treatment with a 0·005 per cent solution of the vesicant was much lower than that produced by a 0·01 per cent solution of the resin, in spite of the longer period of application of the former (Table I). As the yield of vesicant in this experiment was 12 per cent of the resin it is evident that only a small part of the activity of the latter was retained in the vesicant.

This result suggests either that the vesicant and tumour-promoting principles are not identical, or that this sample of the vesicant had lost a large part of its promoting activity during preparation.

It was decided to use Cherbuliez resin, prepared as described in Experimental, Section I, as starting material for further fractionation.

III. *Chromatographic fractionation of Cherbuliez resin.*

In a preliminary experiment the resin (see Experimental, Section I) dissolved in CCl_4 was passed through an Alumina Column. On development a large number of indefinite yellow zones appeared, some of which were readily washed down by weak elutants. A more exact analysis was carried out as follows.

Two grams of resin was dissolved in CCl_4, and deposited on an 80 g. alumina column made up in CCl_4. The column was eluted with successive 100 ml. lots of (1) CCl_4 (2) 10 per cent acetone/CCl_4 (3) 20 per cent acetone/CCl_4.

Eluates (1) (2) and (3) were collected separately, and evaporated on the water bath at 60° C. The column was then broken up into 4 sections, and each exhaus-

tively extracted with methanol. The solvent was evaporated from each, and the 4 extracts numbered 1 to 4, starting from the bottom of the column. Further details are given in Table III.

TABLE III.

Fraction.	Elutant or solvent.	Yield (in grams) from 2 g. of resin.	Yield as per cent or resin.
Eluate 1	CCl_4	0·140	7·0
„ 2	10% acetone/CCl_4	0·023	1·1
„ 3	20% acetone/CCl_4	0·446	22·3
Extract 1	Methanol	0·311	15·6
„ 2	„	0·614	30·7
„ 3	„	0·111	5·6
„ 4	„	0·090	4·5
Total recovery		1·735	86·8

Alumina Column: 80 g. 100 ml. of each elutant 1 to 3. Extracts 1 to 4 were made, after development, from successive sections of the column, starting from the bottom.

In the test for tumour-promoting action, Eluates 1 and 2 were pooled, and so were Extracts 1 and 2, and 3 and 4, respectively. They were applied as 0·02 per cent solutions in acetone, with the exception of Eluate 3 which was applied as a 0·01 per cent solution. The results are given in Table IV.

TABLE IV.

Fraction.	Yields as per cent or resin.	Concentration in acetone applied (%).	Total tumours.	Tumour-bearing mice/survivors.	Tumours per survivor.
Eluates 1 and 2	8·1	0·2	0	0/16	0
Eluate 3	22·3	0·1	47	11/16	3·0
Extracts 1 and 2	46·3	0·2	37	9/16	2·3
„ 3 and 4	10·1	0·2	8	4/16	0·5

Sixteen mice per Fraction: Primary treatment: 0·2 ml. 0·15 per cent D.M.B.A./acetone. Interval: 4½ weeks. Secondary treatment: 0·3 ml. of test solution applied to skin weekly for 13 weeks.

Eluate 3 (20 per cent acetone/CCl_4) and Extracts 1 and 2 (methanol extracts of the lower half of the column after development) contained the bulk of the active agent or agents. These fractions were therefore chosen for further study.

After some further experiment a method was evolved of which the following is an example.

Fifty-four grams of croton oil in 100 ml. of petroleum ether (B.P. 60–80° C.) was shaken with three successive 25 ml. lots of 90 per cent methanol. The latter were pooled, evaporated in partial vacuum at 60° C., and dried in a vacuum dessicator over anhydrous calcium chloride for 1 day. The yield of resin was 1·8 g.

One and a half grams of this resin was dissolved in CCl_4 and deposited on a 60 g. alumina column (Grade II). It was eluted with CCl_4, and mixtures of CCl_4 and acetone containing successively higher concentrations of acetone.

No appreciable amounts of the material were eluted until the acetone concentration reached 20 per cent. Thereafter successive 50 to 100 ml. lots of 20 per cent

acetone/CCl_4 eluate were collected separately, and evaporated as described above. These are listed, and their characteristics recorded, in Table V.

TABLE V.

Fraction.	Elutant.	Volume of elutant (ml.).	Weight of fraction.	Yield as per cent of resin.
1	20% acetone/CCl_4	100	·125	8·3
2a	20% " "	50	·285	19·0
2b	20% " "	50	·191	12·7
3	20% " "	100	·147	9·8
4	20% " "	100	·070	4·7
5	20% " "	100	·044	2·9
6	20% " "	100	·101	6·7
7	20% " "	100	·010	0·7
8	50% " "	100	·006	0·4
9	50% " "	100	·002	0·1
Total recovery			1·035	68·6

Starting material: 1·5 g. resin. Alumina column: 60 g.

Examination of the yield in each fraction shows that the material descends in two main blocks: the first, which is probably complex, comprises Fractions 1 to 5, the second is mainly confined to Fraction 6.

Fraction 1 and Fraction 3, representing the front and rear parts of the first block, and Fraction 6 representing the second block, were separately tested for tumour-promoting action. The results are given in Table VI.

TABLE VI.

Fraction.	Yield as per cent of resin.	Concentration in acetone applied (%).	Total tumours.	Tumour-bearing mice/survivors.	Tumours per survivor.
1	8·3	0·005	98	13/16	6·1
3	9·8	"	28	7/16	1·8
6	6·7	"	0	0/5	0·0

Sixteen mice per fraction. Primary treatment: 0·2 ml. 0·15 per cent D.M.B.A./acetone. Interval: 3 weeks. Secondary treatment: 0·3 ml. of test solution applied to skin three times per week for 9 weeks.

They shew that the activity is carried by the first block of eluted material, and that the front portion of this is more active than the rear.

Having established the general conditions under which an active substance, or group of substances, could be separated from the resin, an attempt was made to split this material into fractions by further chromatography, using graded elutants.

It was found that no further purification could be effected by the use of acetone/carbon tetrachloride mixtures. Methanol/carbon tetrachloride mixtures appeared more promising. To reduce the amounts of liquids to be handled only small quantities of resin were subjected to chromatography. The work was simplified by the discovery that the material eluted from the column gave a reddish yellow colour, in very dilute solution, in presence of concentrated sulphuric acid. The

movement of the bands down the column could be followed by taking samples of eluate at regular intervals, and adding concentrated sulphuric acid.

The following was a typical experiment : ·087 g. of resin, produced as described in Experimental, Section I, was dissolved in about 5 ml. carbon tetrachloride and passed over a 6 g. alumina column made up in carbon tetrachloride.

The column was then eluted, first with CCl_4, then with CCl_4/methanol mixtures containing rising concentrations of methanol (0 to 1·2 per cent), and finally with 20 per cent acetone in CCl_4. Twelve successive fractions were collected. The yields are recorded in Fig. 1.

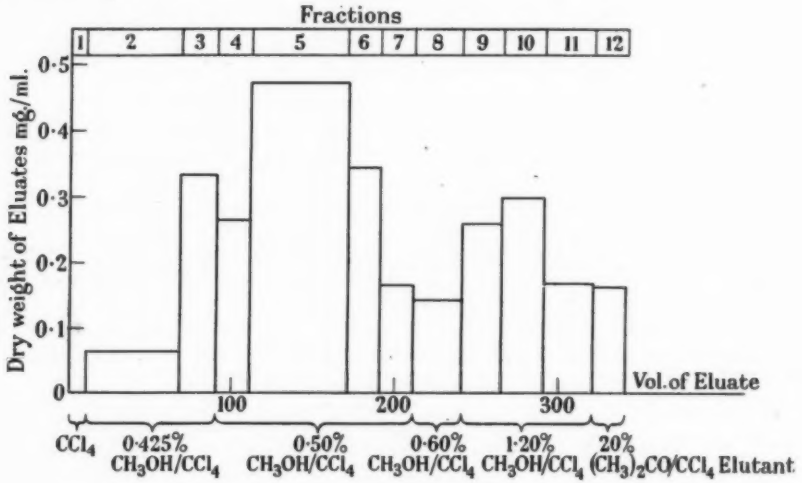

FIG. 1.

There appears to be three peaks. Frequent application of the H_2SO_4 test during elution suggested that a cleaner separation between these peaks had in fact been made than appears from the dry weights recorded. Unfortunately, though this tests reveals very small quantities of these resinous materials, the

TABLE VII.—*Tests of Methanol/CCl_4 Eluates of "Cherbuliez Resin" for Tumour-promoting Activity.*

Sample.	Yield as per cent of resin.	Concentration in acetone applied (%).	Total tumours.	Tumour-bearing mice/survivors.	Tumours per survivor.
1. (Fractions 1 and 2) (see Fig. 1)	18	0·008	0	0/15	0
2. (Fractions 4 to 7) (see Fig. 1)	51	0·008	52	8/15	3·5
3. (Fractions 9 to 11) (see Fig. 1)	17	0·008	0	0/15	0
Resin	100	0·02	35	9/14	2·5

Fifteen mice in each group. Primary treatment : 0·2 ml. 0·15 per cent D.M.B.A./acetone. Interval : 4 weeks. Secondary treatment : 0·3 ml. of sample in acetone applied to skin 3 times a week for 10 weeks.

depth of colour is too much affected by temperature and time of standing to serve as the basis for a useful quantitative colourimetric assay.

Three samples were formed by pooling Fractions 1 and 2, 4 to 7, and 9 to 11, respectively, and tested for tumour-promoting activity. The results are given in Table VII.

It appears that the tumour-promoting activity is confined to Sample 2. Any activity in Samples 1 and 3 must be of a low order.

IV. *Physical and chemical properties of chromatographic fractions.*

Full descriptions of these substances will be given in a later report. A few general properties will be described here.

All the fractions eluted from alumina columns by methanol/carbon tetrachloride mixtures are resinous materials. Attempts to crystallize them have so far been unsuccessful. It is unlikely that any of them is a pure substance. All are insoluble in water and soluble in organic solvents. They give a reddish colour in dilute solution on addition of concentrated sulphuric acid. They are slowly soluble in 20 per cent aqueous KOH. They take up bromide in CCl_4 solution in the cold; further bromine is taken up on heating, with the liberation of HBr. The iodine number determined by the pyridine dibromide method (Hodgson-Jones and Wheatley, 1952) is variable, but is usually about 50 cg. per g. The active fractions (Eluates 4 to 7, Fig. 1) form white friable foamy deposits on evaporation of the solvent, while the inactive fractions have the appearance of very viscous liquids.

All the samples tested for tumour-promoting activity (Table VII) cause hyperplasia of the epidermis of approximately equal severity at similar concentrations.

DISCUSSION.

Berenblum (1944, 1954) has speculated on the nature of tumour-promotion by croton oil and other substances. He points out that of many agents which induce hyperplasia, only a few are tumour-promoting, and therefore that the tumour-promoting action of croton oil cannot depend only on its ability to induce hyperplasia.

However no tumour-promoting agent has yet been discovered which does not also induce hyperplasia of the tissue concerned. Iodo-acetic acid and chloroacetophenone, for instance, previously shown in this laboratory to be tumour-promoting in mouse skin (Gwynn and Salaman, 1953), also induce epidermal hyperplasia. In a recent report Daneel and Wissenfels (1955) describe the separation of croton oil, by a chromatographic method, into irritant and non-irritant fractions. They claim that only the non-irritant fraction is tumour-promoting when applied to mouse skin previously treated with methylcholanthrene. The validity of this claim is difficult to assess because of the small number of mice used, and because the criterion for assessment of the irritant property (entzündlichen Wirkung) is not stated. Their work is being repeated in this laboratory.

As judged by microscopical appearance all the croton oil fractions examined in the present work produced hyperplasia of the epidermis, i.e. this property was not confined to the tumour-promoting fractions.

Further work will be needed before the relation between hyperplasia and tumour-promotion is made clear.

The active fraction which has been separated from croton oil is tumour-promoting in mouse skin in far higher dilution than any other substance known. It is therefore important to determine its chemical nature. Efforts to do this have met with considerable difficulties, but are being continued.

SUMMARY.

The tumour-promoting substance present in croton oil has been concentrated by solvent partition followed by chromatographic analysis. The product obtained, a white amorphous solid, is very much more potent as a tumour-promoter (about fifty times) than the original oil. Some properties of this substance are described.

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THE INITIATING ACTION OF ETHYL CARBAMATE (URETHANE) ON MOUSE SKIN.

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ETHYL carbamate (urethane) was shown by Salaman and Roe (1953) to act as an initiating agent for carcinogenesis, causing mouse skin to become responsive to subsequent croton oil treatment. (The carcinogenic effect of alternate applications of urethane and croton oil to mouse skin, representing a dual co-carcinogenic action by these two agents, was independently demonstrated by Graffi, Vlamynck, Hoffmann, and Schulz (1953) and by Salaman and Roe (1953). According to both groups, urethane alone is non-carcinogenic for mouse skin.)

The present investigation was undertaken to confirm the initiating action of urethane and to gain more information about the number of applications required for this effect to be obtained. Meanwhile, a second communication by Roe and Salaman (1954) appeared, which partly covers the same field.

METHODS.

The animals used were female Swiss mice, inbred in these laboratories. They were housed in an air-conditioned room at 21–22° C, and fed on *Purina laboratory Chow* and water *ad libitum*. A 40 per cent solution of urethane in ethylene glycol was used for initiating action, and a 5 per cent solution of croton oil in medicinal liquid paraffin (in some cases reduced to 2·5 per cent, when the irritating action on the skin was too severe) was used for promoting action. These were applied with a glass rod to an area of skin of about 1 sq. cm. over the shoulder blades, the hair in that region having been removed with scissors before each application. Between the end of the urethane treatment and the beginning of the croton oil treatment, there was an interval of 4 weeks. Records were kept of the resulting skin papillomas (charted when first observed and thereafter at fortnightly intervals), and also of other lesions observed at autopsy, e.g. of lung adenomas and liver lesions, which were kept for histological examination.

RESULTS.

The results, summarized in Table I, bring out the following effects:

(1) Twelve half-weekly applications of 40 per cent urethane in ethylene glycol rendered the skin responsive to subsequent croton oil treatment, as was shown by the development of papillomas at the site of treatment in over 50 per cent of the surviving animals.

(2) A single application of 40 per cent urethane did not produce this effect.

TABLE I.

Number of mice.	Age (months).	Primary treatment.	Secondary treatment.	Mice bearing papillomas/ survivors.*	Times of appearance (weeks).†	Total number of papillomas.	Mice bearing lung adenomas.	Mice with liver haemorrhage.
20	5	Urethane × 1	Croton oil × 70	0/16	—	0	2	2
20	5	Ethylene glycol × 1	„ „ × 70	1/18	20	1	0	0
20	5	Urethane × 1	Liquid paraffin × 70	0/20	—	0	2	1
20	5	Ethylene glycol × 1	„ „ × 70	0/18	—	0	0	0
20	4½	Urethane × 12	Croton oil × 70	10/17	6, 14½, 20½, 21½, 23, 28½, 29½, 32½, 32½, 33½	20	9	3
20	4	Ethylene glycol × 12	„ „ × 70	1/19	32½	3	1	0
20	4	Urethane × 12	Liquid paraffin × 70	0/17	—	0	9	1
20	4	Ethylene glycol × 12	„ „ × 70	0/20	—	0	1	0
20	3½	Urethane × 86	—	0/20	—	0	14	6
15	3½	Ethylene glycol × 86	—	0/15	—	0	0	0

* Survivors : survivors after 6 weeks of croton oil treatment (i.e. the time when the first papilloma appeared in the whole series).
† Time from commencement of secondary treatment.

(3) Urethane alone, applied twice weekly for 43 weeks, did not induce a single papilloma.

(4) Two papillomas (1/18 and 1/19 survivors, respectively) developed in control groups receiving croton oil without previous urethane treatment.

(5) The incidence of lung adenomas was higher among mice receiving 12 or more applications of urethane than in control groups, but was not significantly higher in the group receiving a single painting of urethane.

(6) Lesions of the liver, resembling hepatomas to the naked eye, but proving histologically to be haemorrhages with necrosis of liver parenchyma, were present among the urethane treated animals, and especially frequently among those receiving continuous urethane treatment, but were absent among those not receiving urethane.

DISCUSSION.

These results support the findings of Salaman and Roe (1953) that urethane, itself non-carcinogenic for mouse skin, can induce the initiating phase of carcinogenesis in that tissue. This experimental confirmation of the principle, predicted on the basis of the two-stage mechanism of carcinogenesis (Berenblum, 1952), that tumour production should theoretically be possible by the successive actions of two non-carcinogenic agents—a pure initiator followed by a pure promoter—thus adds to the validity of the two-stage hypothesis. The results are also of technical value for future experiments, since the use of a substance which has an initiating action free from a promoting action has obvious advantages over that of a complete carcinogen in which the promoting action has to be artificially repressed by very short action.

The present results differ in certain respects from those reported by Roe and Salaman (1954), a *single* dose of urethane being found ineffective in our experiments but effective in theirs. Differences in technique were probably responsible for this, e.g. the small area of treated skin (1 sq. cm.) instead of the whole skin of the back, the different strains of mice used, and the fact that the concentrations of reagents and the solvents were not the same.

A surprising feature of the present results was the remarkably long average latent period for tumour induction, compared to that observed when 9 : 10-dimethyl-1 : 2-benzanthracene or one of the other polycyclic hydrocarbons was used as initiator (Berenblum and Shubik, 1947). In the experiments with urethane and croton oil by Salaman and Roe (1953), the latent period was intermediate between the two. No explanation for these divergent results can be offered at present, though it should be pointed out that even with dimethylbenzanthracene as initiator (followed by standard croton oil treatment), different average latent periods have been observed by us in apparently identical experiments carried out on different occasions.

The development of lung adenomas following applications of urethane to the skin (also observed by Salaman and Roe, 1953) emphasize the striking sensitivity of the lung to this agent. [The minimal effective dose by the intraperitoneal route is about 20 mg. (Henshaw and Meyer, 1944)]. The lesions of the liver in urethane treated mice, which appeared by naked eye to resemble hepatomas, proved histologically to consist of extensive haemorrhages with necrosis, *but without any evidence of neoplasia*, contrary to the reports of Salaman and Roe (1953). Damage to the endothelial lining of blood vessels in the liver, with resulting hæmorrhage,

caused by urethane administration, was described by Doljanski and Rosin (1944).

SUMMARY.

The previously reported observation that ethyl carbamate (urethane), though itself non-carcinogenic for mouse skin, is able to induce in that tissue the initiating phase of carcinogenesis, has been confirmed.

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CARCINOGENIC ACTIVITY OF SOME METHOXY-DERIVATIVES OF POLYCYCLIC HYDROCARBONS.

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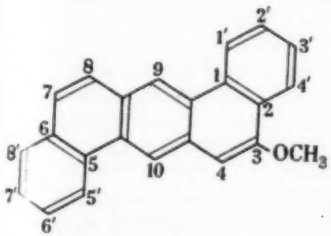
THE finding that 8-methoxy-3 : 4-benzpyrene is one of the most active carcinogens for mice and rats (Cook and Schoental, 1952*b*; Peacock and Head, 1952) stimulated interest in other methoxy-derivatives of polycyclic hydrocarbons. Isomers having the methoxy-group at the more hindered position of the phenanthrenoid bond known as the "K region" seemed of particular interest, as 3-methoxy-1 : 2-benzanthracene has been found to be slightly carcinogenic (Shear and Leiter, 1940). The "K region" may possibly be involved in the metabolic fate of the hydrocarbons (Bhargava, Hadler and Heidelberger, 1955).

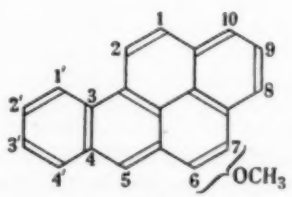
A convenient route for the preparation of these compounds is the osmium tetroxide oxidation of the respective hydrocarbons (Cook and Schoental, 1948) in the presence of pyridine. Hydrolysis of the complexes yields diols, which under the influence of dilute mineral acids undergo dehydration to phenols, methylation of which results in the respective methyl ethers. In the case of 1 : 2 : 5 : 6-dibenzanthracene dehydration of the diol and subsequent methylation yields 3-methoxy-1 : 2 : 5 : 6-dibenzanthracene(I), and only traces of a second isomer. In the case of 3 : 4-benzpyrene two products were obtained, one melting at 136–7° C., the other at 179·5–180° C. Although their respective identity has not yet been proved by independent synthesis, the lower melting compound is likely to be the 7-methoxy-3 : 4-benzpyrene (II), and the higher melting isomer 6-methoxy-3 : 4-benzpyrene(II). Melting points of isomers having a bulky substituent (such as a methoxy-group) at a hindered position are usually lower than the ones substituted at an unhindered position. The compound melting at 136–7° C. was used for biological testing.

In view of the carcinogenic activity of 5-alkyl-1 : 2-benzanthracenes (Barry *et al.*, 1935) 5-methoxy-1 : 2-benzanthracene (III) (synthesised by Cook and Schoental, 1952*a*) was also tested.



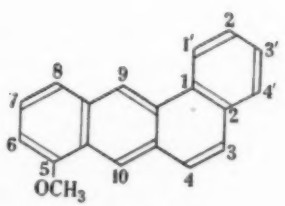
I

3-Methoxy-1 : 2 : 5 : 6-dibenzanthracene.



II

7- (or 6-) Methoxy-3 : 4-benzpyrene.



III

5-Methoxy-1 : 2-benzanthracene.

EXPERIMENTAL.

Mice of mixed stock, bred in the Departmental Animal House, were used. The animals were housed in metal cages, 5–6 per cage, and given Shearer's Pig Weaner Nuts No. 1 (for their composition see Schoental, Head and Peacock, 1954) and tap water *ad libitum*.

I. 3-*Methoxy*-1 : 2 : 5 : 6-*dibenzanthracene*.—Pure crystalline 3-methoxy-1 : 2 : 5 : 6 dibenzanthracene was dissolved in tricaprylin and injected subcutaneously into the left flank of 12 male and 12 female mice, about 6 weeks old; each animal receiving 0·5 mg/0·1 tricaprylin.

II. 7 (*or* 6)-*Methoxy*-3 : 4-*benzpyrene*.—Five male and 10 female mice about 6 weeks old each received a single subcutaneous injection of 0·5 mg. 7 (or 6)-methoxy-3 : 4-benzpyrene in 0·1 ml. of tricaprylin.

III. 5-*Methoxy*-1 : 2-*benzanthracene*. Seven male and 10 female mice about 1–2 months old each received a single subcutaneous injection of 1·5 mg. of pure 5-methoxy-1 : 2-benzanthracene in 0·15 ml. of tricaprylin.

The animals were allowed to live as long as possible: some were killed when moribund. All mice were examined *post mortem*, and the tumours and other observed abnormalities confirmed by histological examination. For this purpose selected tissues were fixed in formol-corrosive, and stained with haematoxylin and eosin. In addition other stains were used in cases requiring further differentiation.

RESULTS.

I. 3-*Methoxy*-1 : 2 : 5 : 6-*dibenzanthracene*.

No tumours were found at the site of injection in any of the 24 mice injected with 3-methoxy-1 : 2 : 5 : 6-dibenzanthracene, although 9 mice survived over 22 months after the injection, and the oily solution remained palpable as a definite nodule under the skin for several months. Lung adenomata were found in 5 females and 2 males. In the livers of 2 female mice (one of which had lung adenomata) hepatomata were present.

II. 7 (*or* 6)-*Methoxy*-3 : 4-*benzpyrene*.

The first tumour at the site of injection with 7 (or 6)-methoxy-3 : 4-benzpyrene was noticed in a male mouse 6 months after the injection. The animal was killed one month later, when the tumour measured 3·7 × 1·8 cm. It was partly haemorrhagic, and partly white and fleshy, and was invading the muscle. No metastases were present. Microscopically the tumour was a pleomorphic sarcoma, composed of round and spindle cells. Some strap-like cells were seen which stained yellow by van Gieson's method. They contained large nuclei, but no muscle striations could be demonstrated in sections stained with phosphotungstic acid, haematoxylin, Heidenhain's iron haematoxylin or Celestial Blue. In places many giant cells were noted and mitoses were numerous. The tumour was seen invading muscle. Among the remaining 4 male mice one had lung adenomata, one had a tumour of the thymus and one lymphomatosis.

Among the female mice, 3 developed tumours at the site of injection after 6, 11 and 14 months respectively. On microscopic examination these proved to be sarcomata of the spindle cell type.

No neoplastic changes were found in the remaining 7 females and 1 male.

III. 5-*Methoxy*-1 : 2-*benzanthracene*.

In 5 of the 17 mice, 4 females and 1 male, tumours developed at the site of injection of 5-methoxy-1 : 2-benzanthracene. The first tumour appeared about 16 months after the injection in a female mouse. The animal was killed about 3 weeks later, and had a white fleshy tumour 1·5 × 1 cm. on the left side of the back, invading muscle. Microscopically it proved to be a spindle cell sarcoma in which numerous giant cells were present. Many cells were in mitosis. In the liver of this animal some zonal degeneration and regeneration were present. In the regenerating areas mitoses were seen in a number of cells. Similar tumours, spindle cell sarcomata, were present in 3 other female mice killed 17 months, 21·5 months and 24 months after the injection, respectively. Lung adenomata were found in one tumour-bearing mouse killed 17 months after the injection, as well as in one female mouse which died 25 months after the injection and which had no local tumours.

Among the 7 male mice of this series only one was found to have a tumour 2·4 × 2·4 cm. at the site of injection when the animal was killed after 21 months. On microscopic examination the tumour proved to be a spindle cell sarcoma invading muscle. In the liver of this animal a hepatoma containing many eosinophil cytoplasmic inclusions was present in the upper part of the central lobe.

No tumours were found in the remaining 12 animals of this series.

DISCUSSION.

It is of interest that 3-methoxy-1 : 2 : 5 : 6-dibenzanthracene did not produce any tumours as carcinogenic activity has been demonstrated in the case of 3-methoxy-1 : 2-benzanthracene (Shear and Leiter, 1940). No such difference was shown by methoxy-derivatives of these two hydrocarbons, having the substituent at the meso-position. Thus both 9-methoxy-1 : 2 : 5 : 6-dibenzanthracene and 10-methoxy-1 : 2-benzanthracene produced tumours in mice : the former when tested by repeated subcutaneous injections (Shear and Leiter, 1940), the latter by applications to the skin of a 0·3 per cent, solution in benzene (Barry *et al.*, 1935).

7 (or 6)-methoxy-3 : 4-benzpyrene produced sarcomata in 4 out of 15 animals, 6–15 months after the injection. Thus, this isomer is a less potent carcinogen than 8-methoxy-3 : 4-benzpyrene, which gave tumours in 8 out of 16 mice 10-14 weeks after subcutaneous injection of a similar dose. Its activity is comparable with that of 5-methoxy-3 : 4-benzpyrene, which produced tumours in 2 out of 10 mice after subcutaneous injection in the course of 12 months (Berenblum and Schoental, 1944).

5-Methoxy-1 : 2-benzanthracene gave rise to 5 sarcomata in 17 mice ; the tumours appearing 16–24 months after the subcutaneous injection. This long latent period indicates that this compound is a weak carcinogen ; the corresponding 5-methyl-1 : 2-benzanthracene produced 5 epitheliomata and 2 papillomata in 10 mice receiving skin applications of a 0·3 per cent solution in benzene twice weekly (Barry *et al.*, 1935) in an experiment lasting 433 days.

No significance can be attached to the occurrence of lung adenomata or liver tumours in the experimental animals. A fairly high incidence of these tumours, especially of lung adenomata, having been encountered among control mice of the mixed strain used.

We wish to thank Dr. P. R. Peacock, the Director of Research, for his interest, the technical staff of the Pathology Department for histological preparations and

the staff of the Animal House for care of the animals. This work has been supported by a grant from the British Empire Cancer Campaign.

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EXPERIMENTAL CIGARETTE SMOKING BY DOMESTIC FOWLS.

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EVIDENCE of an association between lung cancer and a history of heavy cigarette smoking has been reported both in Great Britain and in the United States of America (Doll and Hill, 1954 ; Wynder and Graham, 1950). It does not follow that heavy smoking necessarily entails an increased risk of lung cancer, since there may be great differences in individual susceptibility to the causal factors concerned, whose nature has not yet been established. Moreover, residence in towns seems also to be a possible aetiological factor in men who have lung cancer. Thus cigarette smoke and atmospheric pollution are extrinsic factors that should be investigated for possible carcinogens or co-carcinogens.

Experimental smoking by animals seems to be an obvious method of testing for carcinogenic agents in cigarette smoke, but there is great difficulty in devising a technique that resembles the human habit. Mice or other animals enlcosed in a confined space into which cigarette smoke is blown resemble non-smokers in a smoking compartment rather than smokers, and, like them, seem to find the experience disagreeable. Such animals are unable to breathe relatively fresh air between puffs of smoke as is done by human smokers, and must have their fur impregnated with smoke products which they lick when grooming themselves. Thus such experiments cannot be said to resemble closely the human habit.

The desirable criteria for a cigarette smoking test seem to be (*a*) that the smoke should be drawn into the lung of the animal without traversing a free air space in which cooling and precipitation of substances may occur ; (*b*) that the inhalation should be intermittent with access to relatively smoke-free air between puffs as in human smoking ; and (*c*) the distance travelled by the smoke between the cigarette and the lung should be about 12 inches as in the human subject. These criteria can be met by using the air sacs of fowls as a means of access to their lungs.

The anatomy of the bird's lung differs considerably from that of mammals, but serves the same physiological function and thus seems a suitable test for exposure to smoke. Moreover, spontaneous lung cancer seems to be rare in birds, in which, however, we were able to induce bronchial carcinoma with 2-acetyl laminofluorene (Peacock and Peacock, 1954). The existence of air sacs has been known for a very long time and many physiological studies have been made of the respiration of birds (Graham, 1939 ; McLeod and Wagers, 1939). The available evidence shows that most of the respiratory exchange in birds occurs during expiration when the air drawn through the bronchi into the air sacs during inspiration is passed back through the recurrent bronchi into the air passages

of the lungs. Thus smoke introduced into an air sac must traverse the lung before emerging through the mouth.

Preliminary injection of a liquid plastic which polymerises within 24 hours, through the trachea of freshly killed birds permitted a detailed study of the air sacs to be made. The transparent casts of abdominal air sacs, are illustrated in Fig. 1. There are also sacs in the axillary region running through the shoulder joint into the shaft of the humerus. They are well seen during the dissection of the injected specimen, but are more difficult to illustrate because of their tortuous course which makes the casts fragile and difficult to preserve.

After vigorous flapping of the wings the axillary air sacs can be seen pulsating just beneath the skin in the axilla over the shoulder joint. At this point an opening 5 mm. long was made, under ether anaesthesia, and the cut edges of the air sac were stitched to the skin with fine cotton thread. The resulting stoma was used four days later for introducing a glass canula attached to a simple smoking apparatus as illustrated (Fig. 2 and 3). This consists of an all-glass 20 ml. syringe and a Y-shaped pyrex glass tube with glass non-return valves in the two limbs not connected to the syringe. A cigarette inserted into one limb of the apparatus can thus be smoked by means of the syringe used as a pump.

The rate of smoking and the volume of each puff can easily be controlled by the operator. After some trials a routine was established as follows: 15 ml. of smoke are drawn slowly into the syringe so that the cigarette end just glows (in diffuse daylight). The smoke is then expelled steadily through the stoma into the lung of the fowl whence it emerges a second or two later through the mouth (Fig. 4). The bird is allowed to take five breaths before a second puff is injected in the same way and this process is repeated until fifty breaths have been taken. Thus on average ten injections each of 15 ml. of smoke are used during the smoking session. In this way the bird breathes, in and out, smoke with intermittent free air breathing as in human smoking.

At first exposure all birds seemed to experience malaise similar to that experienced by human smokers, and there seemed to be some variation in the severity of the symptoms. Some birds merely showed surprised interest in the smoke emerging from their beaks; others closed their eyes and drooped their heads, evidently disliking the experience. When this happened the exposure was stopped for the day.

Birds quickly became accustomed to the procedure and after a few days showed no signs of discomfort and accepted their smoke without opposition.

EXPLANATION OF PLATE.

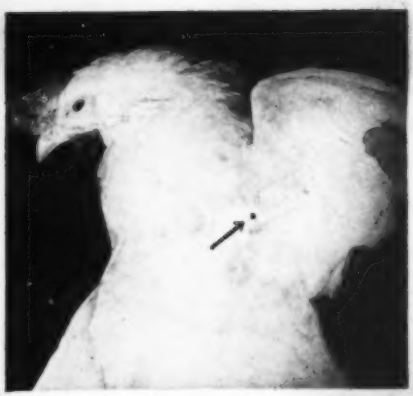
FIG. 1.—White Leghorn hen. Trachea, main bronchi, lungs and abdominal air sacs injected with plastic. Viewed from behind. The abdominal viscera have been removed.

FIG. 2.—White Leghorn hen No. 4317, showing stoma (indicated by arrow) from left axilla to thoracic air sac.

FIG. 3.—All-glass pumping system for smoke injection: (i) and (ii) ground glass non-return valves; (iii) 20 ml. all-glass syringe; (iv) No. 19 stainless steel serum needle. With this apparatus no preliminary operation is necessary, as smoke can be injected directly through the skin into the thoracic air sac in the axilla.

FIG. 4.—White Leghorn fowl No. 4317. Smoke is emerging from the beak during injection through the stoma shown in Fig. 2.

1



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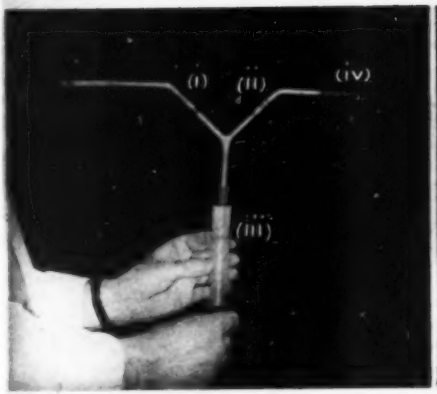

3

4

Peacock.

Owing to a tendency for the stoma to heal after about fourteen days, a simpler procedure is now employed. A short, wide-bored, stainless steel needle (No. 19 serum) fitted to the outlet of the smoking apparatus is thrust directly into the axillary air sac through the skin. When smoke is drawn into the syringe it can be seen oscillating back and forth in the Y-tube with each breath if the needle is in the sac; thus the risk of causing surgical emphysema can be avoided when injecting the smoke. At the time of writing four birds have been smoking half a cigarette each every other day for three months with no obvious ill effect. This is taken only as evidence that the dose is not immediately toxic and that the experiment can be carried on for a reasonably long period. As domestic fowls have lived for up to ten years in our experimental farm laboratory, it is probable that the test will have to be continued for several years before the effects can be assessed.

SUMMARY.

A simple procedure for injecting cigarette smoke into the thoracic air sacs of fowls is described.

The smoke passes through one lung and out through the mouth and is partly re-inhaled, so that the whole respiratory system is exposed to the smoke.

The smoke inhalation is intermittent as in the human habit. Tolerance is quickly established and maintained at half a cigarette per bird every other day.

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MOUSE CAGES AND SPONTANEOUS TUMORS.

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THE investigator who is engaged in research on laboratory animals frequently is faced with problems of animal maintenance, and, on occasions, is required to make decisions concerning the animal cages. Within certain obvious limits, the cage type probably is irrelevant to the outcome of most experiments of short duration. However, it is conceivable that some results of long-term experiments might vary with different kinds of cages. The purpose of the present study was to determine whether mice that spend their lives in plastic cages are, in any easily measurable way, different from mice that live in metal cages.

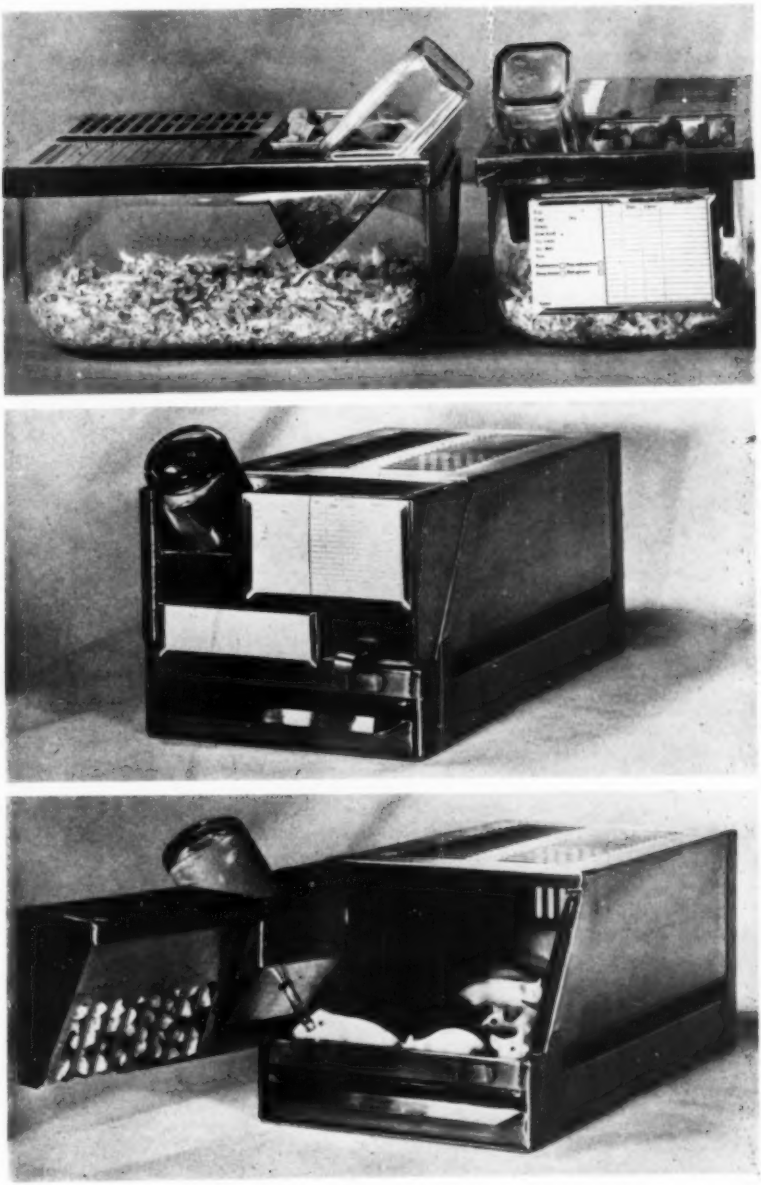
MATERIALS AND METHODS

The two cages are pictured in Fig. 1. All parts of the metal cage and the cover of the plastic cage were constructed of 302-304 stainless steel; the body of the plastic cage was made of HC202 Lucite, a methyl methacrylate polymer. The living space in the metal cage was $14 \times 7\frac{1}{2} \times 6$ inches high and in the plastic cage it was $10\frac{1}{2} \times 6\frac{1}{4} \times 4$ inches high. Fifteen animals per metal cage and ten per plastic cage resulted in 7·0 and 6·6 square inches of floor space per mouse, respectively. Major features of the plastic cage, in addition to the smaller number of animals living in competition and in increased illumination, are the absence of cross-ventilation and the presence of sterile wood shavings as a bedding material.

A single shipment of 375 five to six-week-old CF No. 1 female mice were distributed according to a table of random numbers into 15 metal cages (225 animals) and 15 plastic cages (150 animals) immediately upon arrival in the laboratory, and each mouse spent the remainder of its life in the type of cage to which it originally was assigned. Clean metal cages were provided once a week, and the plastic bottoms were replaced twice a week. Pelleted food and water were constantly available. The mice were observed daily, and once a month each animal was examined individually. They were autopsied after natural death or after being killed with Nembutal® when in a moribund state. The liver, kidney, spleen, mesenteric lymph node, and thymus, as well as any unusual tumors, were prepared for histological examination, and skeletal roentgenograms were studied.

EXPLANATION OF PLATE.

FIG. 1.—The plastic and metal cages.



Finkel and Scribner.

RESULTS.

Body weight.

The day after they arrived at the laboratory the average body weight of the animals in plastic cages was 0·4 g. greater than that of the animals in metal cages. This difference had become 1 g. by the 28th day and 2 g. by the 237th day, at which time the mice were approximately 280 days old (Fig. 2). This variation in average weight persisted, but it was not statistically significant since the standard deviation in each group was so great.

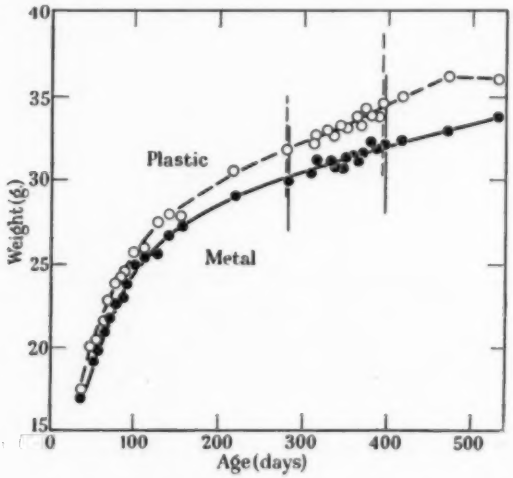


FIG. 2.—Average body weights of the two populations. The vertical lines include one standard deviation.

Survival.

During the first year 20 of the 225 animals in metal cages died of acute respiratory or intestinal infections ; no similar deaths occurred among the 150 animals in plastic cages. A graphical comparison of these percentages on binomial probability paper (Mosteller and Tukey, 1949) indicated that this difference probably was not due to chance ; a similar analysis of the proportions of survivors at 360 days showed that the difference had a 5 per cent probability of being due to chance. When these 20 animals are excluded from the calculations, the life expectancy and mortality rate curves for the two populations are similar (Fig. 3 and 4, respectively).

Tumors.

The original number of animals was reduced in the course of the experiment by a few accidental deaths and escapes, and some animals were excluded from further consideration because their tissues were too autolyzed for histological study. Consequently, the tumor analysis was based on 198 animals that lived in metal cages and 139 that lived in plastic cages.

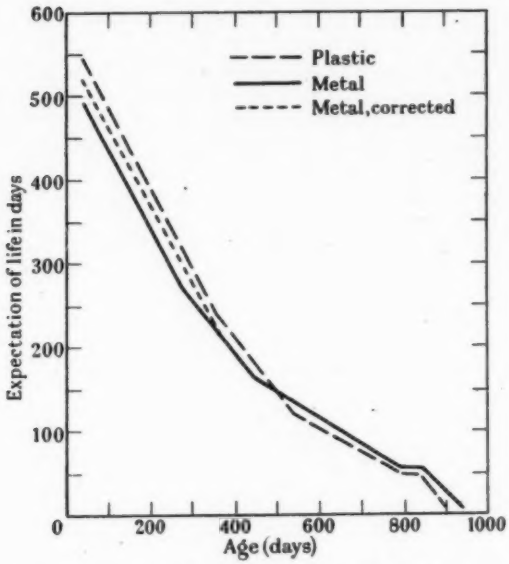


FIG. 3.—Life expectancy. Correction is based on exclusion of animals that died of acute respiratory or intestinal infection during the first year.

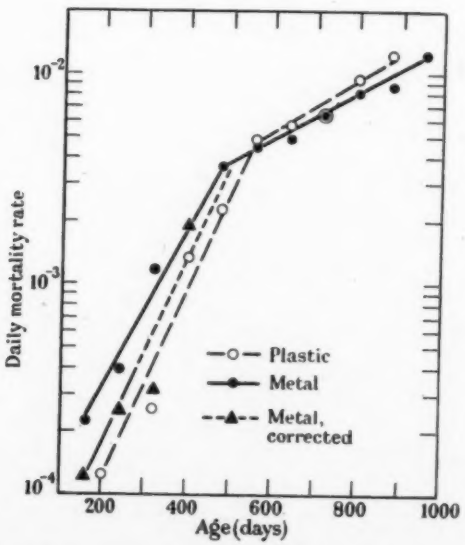


FIG. 4.—Mortality rate. Correction is the same as that applied in Fig. 3.

The tumors were grouped according to the organ or tissue of origin; no distinction was made between benign and malignant growths. Mammary tumors, lung tumors, and tumors of the reticular tissues (primarily thymus, spleen, lymph nodes and Peyer's patches) were considered separately. All the others, which included neoplasms of the skin, liver, skeleton, stomach, uterus, vagina, adrenal gland, harderian gland and parotid gland, were placed in a fourth, miscellaneous category. Ovarian tumors were excluded since the gonad had not been taken routinely for histological study, and the differentiation of neoplastic and non-neoplastic ovaries requires microscopic examination. Some animals possessed two or more similar tumors; those with multiple lung tumors were counted as a

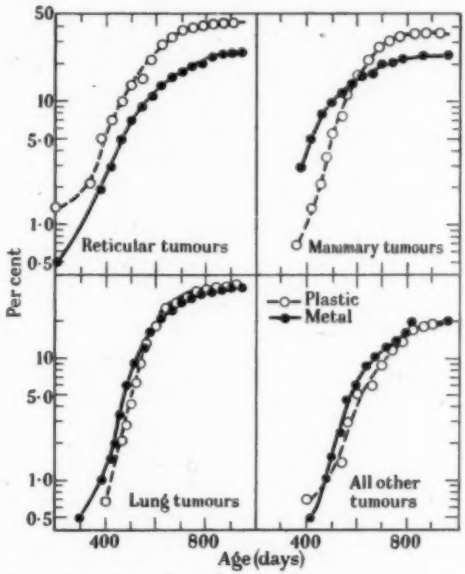


FIG. 5.—Cumulative tumor incidence :

$$\frac{\text{Total number of cases}}{\text{Total number of animals}}$$

single unit since no attempt had been made to list every pulmonary neoplasm, whereas those with other types of multiple tumors were given a unit value equal to the number of tumors.

Early in the experiment tumors of the reticular tissues appeared more frequently among animals in plastic cages, and the cumulative incidence continued to exceed that observed among animals in metal cages (Fig. 5). The difference between the final proportions of animals with reticular tumors in the two populations was 3·7 times as great as the standard error of the difference; the probability that this was a chance difference due to sampling is 1 in 5000. These neoplasms were classified according to the criteria of Dunn (1954), and it was found that the entire group rather than a specific type was involved in the increase. In the metal

cages 47 per cent of the reticular tumors were lymphocytic, 31 per cent were reticulum cell sarcomas type A, 16 per cent were reticulum cell sarcomas type B, and 6 per cent were stem cell, granulocytic, or plasma cell neoplasms. The corresponding percentages in the plastic cages were 44, 26, 16 and 14.

The total incidence of mammary tumors also varied in the two kinds of cages ($P = \cdot014$), but the incidences of lung tumors and miscellaneous tumors were similar ($P > \cdot5$). In addition, the lung and miscellaneous daily tumor rates were the same in the two populations, but the reticular and mammary tumor rates were dissimilar (Fig. 6). The tumor expectancy curves in Fig. 7 demonstrate in still another way that the expression of lung tumors and miscellaneous tumors was the same whether the animals lived in plastic or in metal cages, but that the expression of reticular tumors and mammary tumors was associated with cage type.

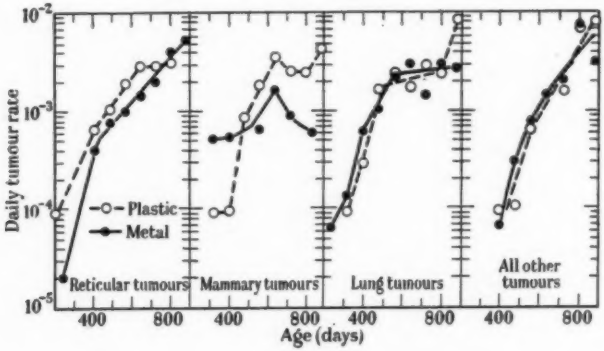


FIG. 6.—Daily tumor rate :

$$\frac{\text{Number of cases autopsied during 80-day interval}}{\text{Number of animals alive at beginning of interval}} \times \frac{1}{80}.$$

It is interesting that the four groups of neoplasms display four different sets of characteristics. These involve age at the time of death of the first tumor-bearing animal, total incidence, and change in tumor rate with increasing age and in the probability of such a tumor being present at death. For example, although the lung and miscellaneous tumor rates have similar slopes for 300 days, the former originates approximately 120 days earlier and levels off before 600 days of age, whereas the latter continues to increase as long as any animals remain alive (Fig. 6). Both groups of tumors show relatively constant expectancies up to 300 days of age and increased expectancies at 500 days (Fig. 7). However, the probability of having a lung tumor at death does not change after an animal reaches approximately 500 days of age, while the probability of having one of the miscellaneous tumors continues to increase.

Since the total tumor incidence was greater among animals in plastic cages, and since some mice possessed as many as five tumors, the question of whether or not the neoplastic events were independent of one another became pertinent. For each population the observed frequency of animals with 0 to 5 tumors was

compared to the expected frequency for a Poisson distributed variable with means equal to the observed avarage number of tumors per animal (1·1 in metal cages and 1·4 in plastic cages), and in each there were more animals with one or two tumors than could be accounted for by chance. This result prompted a comparison of the number of times that various combinations of tumor types appeared in the same animal with the number of times this could be calculated to happen on the basis of the total observed frequencies of the various tumors. It was found that the combinations occurred in metal cages an average of only 76 per cent and in the plastic cages only 52 per cent as often as they would be expected to occur.

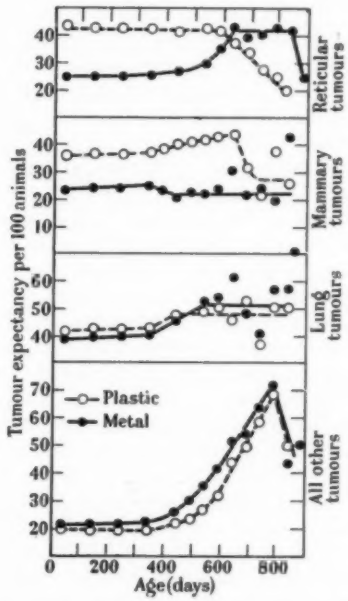


FIG. 7.—Probability per hundred animals of possessing tumor at death : $\frac{\text{Number of cases to come}}{\text{Number of animals still alive}} \times 100.$

This meant that 24 per cent of the animals in metal cages and 48 per cent of those in plastic cages that already had one tumor were somehow prevented from getting another. There was a suggestion that this happened most frequently when reticular tumors were involved, but there was no evidence that a specific type of tumor inhibited another type. It appeared, rather, that some animals died of an already existing tumor before the next one could arise. When this correction factor was applied to the observed frequencies in the metal cages by assuming that 24 per cent of the animals that had one tumour should have had two, that 24 per cent of the new total with two should have had three, etc., the average number of tumors per animal became 1·3, and the hypothesis that the corrected frequencies of occurrence were compatible with the Poisson expectations proved

tenable with probability value of ·65. The correction of 48 per cent similarly applied to the observed frequencies in the plastic cages resulted in a new mean of 2·2 tumors per animal and a probability of 50 out of 100 that the differences between corrected and expected frequencies are zero. This correction magnifies the difference in tumor incidence between the two populations; whereas the standard error of the difference between the two observed means is 0·106 tumors, which gives a t value of 2·84 and a P of 0·4 per cent, the corresponding values of the corrected means are: $\sigma_D = 0{\cdot}158$, $t = 5{\cdot}73$, $P = < {\cdot}0000005$.

DISCUSSION.

The present experiment sheds no light on which of the several dissimilar environmental factors is responsible for the observed variations in tumors of the reticular tissues and mammary glands associated with living in metal or in plastic cages. It is doubtful that the different amounts of light reaching the animals be the causative factor, or that the wood shavings or the plastic itself be carcinogenic under these conditions. However, the latter should not be discounted as a possibility since Oppenheimer, Oppenheimer and Stout (1952) have reported the induction of sarcomas in rats and mice by cellophane, polyethylene and polyvinyl chloride films implanted subcutaneously, and Laskin, Robinson and Weinmann (1954) obtained similar results in mice with methyl methacrylate implants.

The other major differences between the metal and plastic cages contribute to what would seem to be an easier life in the latter. These are: (*a*) a more natural habitat provided by a substrate of wood shavings, (b) reduced competition since there was a cage population of 10 rather than 15, and (*c*) reduced loss of body heat because of the lack of cross-ventilation, the low heat conduction of Lucite and the presence of a bedding material. These apparent advantages of the plastic cage were associated with a decreased incidence of acute lethal infections of the respiratory and digestive systems during the first year of the experiment. Although hundreds of mice of other experiments have been housed in metal cages without the appearance of similar infections, mice living in plastic cages have had such infections. It seems that in this particular shipment of animals the level of endemic diseases was such that they did not become overt among animals in plastic cages, where living was easy. However, in metal cages, where the environmental stresses were greater, a small proportion of the population developed active symptoms of disease.

The consistently greater average body weight of the animals in plastic cages suggested a possible association of body weight and tumor incidence. It has been shown that the number of spontaneous lung tumors, mammary tumors and leukemias in mice decreases when caloric intake is restricted (Saxton, Boon and Furth, 1944; Tannenbaum, 1940, 1942; Visscher *et al.*, 1942) and that mammary tumors appear earlier among animals that are made obese experimentally (Waxler, 1954; Waxler, Taber and Melcher, 1953). No correlation was found, however, between the weight of an animal at 280 days of age and its ultimate neoplastic history in the present experiment. This age was selected since at that time the individual mice were well established in a weight class and they were still relatively healthy, so body weights were not influenced by wasting diseases or by massive tumors.

Andervont (1944) has reported that C3H mice caged individually develop

mammary tumors earlier than their littermates caged in groups of eight. He also noted that the segregated animals were somewhat heavier than the non-segregated animals, but the difference was not considered to be significant. In his experiments any differences were attributable to the number of animals living together, since only one cage type was used. It has been suggested by Rusch (1944) that forcing a mouse to do physical work would be an indirect way of effecting caloric restriction. Imposing cage mates upon a mouse is one way of forcing it to perform additional physical labor, since it must compete for food, water and nesting site. One result of social stimulation also would be an increase in energy expenditure. In the present experiment the competitive environment involves ten versus fifteen animals, which may have been an important factor in determining the results. Of equal or even greater importance, however, is the probability that the maintenance of body temperature required a substantially greater expenditure of energy by mice in metal cages than by mice in plastic cages.

It is hoped that further experimentation will elucidate the environmental factor or factors responsible for the observed results. The present study illustrates the importance in long-term experiments of housing control and experimental mice in similar cages, and indicates that errors of interpretation might result when studies involving animals maintained in different kinds of cages are compared.

The lack of similarity among the four groups of spontaneous neoplasms with respect to the time of their first appearance, incidence, rate and expectancy indicates the existence of fundamental differences beween them. They may appear early in life or late, involve only a small proportion of the population or the majority, and display an ever-increasing, a constant, or a decreasing rate and probability of occurrence with increasing age. Even the response to the "cage factor" varied. Tumors of the reticular tissues appeared earlier in the plastic than in the metal cages, but in the former the rate became constant and the expectancy decreased after 600 days of age. These results suggest that the number of animals capable of possessing such tumors was limited. Mammary tumors, on the other hand, seemed to be repressed in plastic cages before 400 days of age, but the greatly accelerated rate thereafter soon resulted in a greater occurrence than in metal cages. The rate and expectancy curves suggest that the potential incidence of mammary tumors was not limited to the same extent as the potential incidence of reticular tumors. This may be due to the fact that no animal was listed as having more than one reticular tumor, since this would be a difficult diagnosis to establish, while the presence of more than one breast tumor was relatively common and unequivocal.

The distribution of tumors within each population was shown to be random when appropriate corrections were made for the non-survival of a portion of the tumor-bearing animals. The correction appeared to be justified, since some tumors killed rapidly before more could arise, others that were equally lethal in the long run progressed more slowly, while many that were present at death were merely incidental and did not contribute to the morbidity of the animals. Tumors of the reticular tissues most often seemed to be responsible for eliminating animals before additional neoplasms could appear. The random appearance of all types of spontaneous tumors among animals exposed to similar risk indicates that there are neither especially tumor resistant nor tumor susceptible CF No. 1 female mice and that the various types of tumors are independent of one another.

SUMMARY.

CF No. 1 female mice were housed in stainless steel or in methyl methacrylate cages from approximately 40 days of age until death. Although the average body weight of the animals in plastic cages was consistently greater, the difference was probably not significant. Differences in mortality rates and in life expectancies during the first year were ascribable to the death from acute respiratory or intestinal infections of 9 per cent of the animals in metal cages only.

The animals in plastic cages had a higher total incidence of tumors of both the reticular tissues and mammary glands. The differences between the two populations were further illustrated by the dissimilarities in the age-dependent tumor rates and expectancies. However, the expression of lung tumors and all other tumors combined was the same in the two populations.

Many animals possessed more than one tumor; the only restriction to the random distribution of neoplasms among animals exposed to similar risk was that some mice presumably failed to develop additional neoplasms because death from one already present intervened.

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The authors are indebted to Dr. Thelma Dunn of the National Cancer Institute for her assistance with the classification of many of the more difficult tumors of the reticular tissues and to Ruth Bansch, Juanita Lestina and Patricia Sieck for their technical aid.

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CATHEPTIC AND DIPEPTIDASE ACTIVITIES OF ASCITES TUMOUR CELLS.

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In the course of quantitative studies on the topical distribution of some proteolytic activities in malignant tissues, it became of interest to investigate also the proteolytic activities of ascites tumour cell populations, which allow calculations of enzyme activities on a per cell basis. The *in vitro* " available " cathepsin and dipeptidase activities of three different ascites tumour series will be reported. Present data express enzymic activities (without added activators) under aerobic conditions and data are correlated to the duration of growth after inoculation. This study covers only limited aspects of an admittedly complex field of research. We lack, moreover, full information on the possible presence of enzymic activators and inhibitors.

MATERIALS AND METHODS

Mice.

Commercial heterozygous albino female mice, 2 to 3 months old and weighing 20 to 22 g. were used. The animals were kept on a standard diet and got food and water *ad libitum*.

Tumours.

Series of the tetraploid Ehrlich, the hyperdiploid " Landschütz " variety of Ehrlich, and the nearly tetraploid Krebs 2 ascites tumours have been studied. The Landschütz variety was characterized by a lower frequency of haemorrhages than the other strains. These tumours have been maintained by serial ascitic transfers in hybrid mice by injecting 0·1 to 0·2 ml. of non-diluted ascitic fluid intraperitoneally.

Procedure.

Three separate series are included in this report. In each case 5 to 7 days after the inoculation of 0·2 ml. non-diluted ascitic fluid the ascites of 2 or 3 mice was collected and pooled. Part of the fluid was used for cell counting and part for fresh smears that were fixed and stained according to the Papanicolau method. Thereafter, dilutions were made with Krebs-Ringer phosphate (pH 7·3) and $1{\cdot}8 \times 10^6$ tumour cells were inoculated intraperitoneally into each one of 18 to 30 mice. From the second or third day after inoculation, 2 to 4 mice were sacrificed daily and their ascitic fluid was removed for investigation. Samples with marked blood

admixture were discarded. In one series of Krebs 2 tumour the total number of ascites cells was determined by the dye-dilution method of Révész and Klein (1954). The proportion of tumour cells was obtained by differential counts.

In order to obtain samples for the determination of the proteolytic activity of non-tumorous exudate cells, a small volume of saline was injected intraperitoneally into normal mice. The fluid was immediately removed and the concentration of cells was determined, and then the suspension was centrifuged. Enzyme activity measurements were carried out on the washed and lysed sedimented cells as described below. Further, it became necessary to assess the influence of admixed red blood cells appearing in some ascites tumour samples as a sequence to intraperitoneal haemorrhages. Activity measurements on washed and lysed normal red blood cells from the same commercial batch of mice are reported below.

Extraction of ascites cells.

Ascites fluid containing a known number of tumour cells was centrifuged and washed twice in 2 per cent NaCl solution in order to remove the ascites fluid and the dipeptidase inhibitor mentioned below. The washed cells were lysed and homogenized by means of a small tissue grinder in 0·05 per cent sodium desoxycholate. At this concentration cholate has no inhibitory effect on the proteolytic enzymes of the ascites cells according to our findings. The homogenate was diluted with glycerol-phosphate buffer (pH 6·8, glycerol concentration 25 per cent, ionic strength 0·01) until a final concentration usually corresponding to 8×10^6 cells per ml. solution. After extraction for 2–3 hours, the cell débris was centrifuged off and the extract was stored at 0° C.

Protein determinations.

Throughout the Landschütz series, the total amount of protein present in the ascites cell homogenates was determined according to the method described by Nayyar and Glick (1954). The Kjeldahl method for nitrogen determination was used in the Ehrlich and Krebs 2 series.

Determination of dipeptidase activity.

The dipeptidase activity was determined according to the microtechnique developed by Linderström-Lang and Holter (1932). 10 μl. of 0·2 M solutions (containing sodium hydroxide to give the pH mentioned in Table I) of various peptides were incubated at 40° C. with the tumour extracts (7 μl.) and after a suitable time (30–180 minutes) the liberated —COOH groups were determined by formol titration with 0·10 M NaOH using a mixture of phenolphtalein and thymol blue as indicator (Engel and Heins, 1947). Under these conditions, pilot experiments showed a linear relationship between relative dipeptidase concentrations and NaOH consumption up to 9 μl. (cp. Holter and Løvtrup, 1949).

Determination of catheptic activity.

The micromethod of Duspiva (1939) was applied using 1 per cent solutions of nitrocasein or edestin. 10 μl. of the substrate solutions in acetate buffer at pH 5·3 and 3·8 respectively, were incubated with 5 μl. of the cell extracts at 40° C. After suitable time, the reaction was stopped with 5 per cent trichloroacetic acid

(20 μl.), which precipitated the non-digested substrate. The precipitate was then spun down. When using nitrocasein as substrate, an aliquot (20 μl.) of the supernatant was brought to alkaline pH with NaOH (20 μl.). The amounts of coloured split products were then determined in a Photovolt microcolorimeter at 420 mμ. This instrument was of the same design as described by Holter and Løvtrup (1949), and Krugelis (1950). In the case of edestin we added Folin-Ciocalteu's reagent to a similar aliquot (20 μl.), and the amount of split products was determined in a Beckman Model DU, using microcuvettes.

RESULTS.

The cells in the ascitic fluid represent a mixed population of tumour, mesothelial, white blood cells, and macrophages. In all cases, differential counts indicate that after about two days following inoculation of about $2 \cdot 10^6$ cells, the average percentage of tumour cells amounts to approximately 85 per cent of the total ascites cell numbers (Klein, 1951).

Protein increase during tumour growth.

The observed figures for total protein content of tumour homogenates calculated per 10,000 cells are plotted in Fig. 1, A and B. From the 2nd until about the 8th day after inoculation there was a gradual increase in amount of total protein per cell in both Landschütz and Krebs 2 series. After this time the protein content per cell remained at a fairly constant level.

Proteolytic activity of the ascites fluid and the non-tumorous peritoneal cells.

The dipeptidase and catheptic activities of the cell-free ascites fluid obtained in this series of ascites tumours were very low. Both activities expressed per microgramme of protein only amounted to about 5 per cent of those of the tumour cells, and showed no significant variations during tumour growth.

The dipeptidase activity of the non-tumorous peritoneal cells obtained by washing the normal peritoneal cavity with saline amounted only to about 0·2 μl. NaOH per 10,000 cells per hour, using AlaGly as substrate. Since the ratio of tumour cells and non-tumorous cells was constant from the third day after inoculation, it was assumed that variations in enzyme activity of the entire population were due to variations in the enzyme activity of the tumour cells *per se*. This is true under the provision that the non-tumorous exudate cells do not vary their composition and types during the course of tumour growth. The catheptic activity of these exudate cells was also negligible.

Red blood cells are regularly found in many ascites tumours towards the end of their growth period but this cannot explain the observed increase in dipeptidase activities to be reported below. The dipeptidase activity of washed and lysed normal red cells from the commercial mice was found to amount to about 1 μl. 0·1 M NaOH per 10^6 cells per hour (AlaGly ; pH 7·7). On the other hand the catheptic activity was very low, amounting to about 2 per cent substrate degradation per 10^6 cells per hour (edestin ; pH 3·8). Since all tumour samples with macroscopic admixture of blood were discarded, a smallish admixture of blood in cases accepted for investigation can only explain a small part of the observed dipeptidase activities, and will not interfere with the main results.

Proteolytic activity of washed ascites.

Tumour Cells.—In all series of investigated ascites tumours a gradual increase in the *average dipeptidase activity* per tumour cells was found parallel with the time after inoculation (Fig. 2, upper curves). Similar increase in dipeptidase activity was noted when the observed figures were plotted against the total tumour cell numbers after inoculation (data not reported in detail). Counted on a protein basis, however, the dipeptidase activity was fairly constant throughout the growth of the tumours (Fig. 2, lower curves). In one series of ascites tumours the dipeptidase activity of the tumour cells was tested at a number of stages of tumour growth against several substrates (Table I). With all substrates tested, except GlyTry, a similar increase in the average dipeptidase activity per cell was found with the time passed after tumour inoculation. The most suitable substrate seemed to be AlaGly, which was then used as single substrate in the other series.

TABLE I.—*Dipeptidase Activity of Krebs 2 Tumour Cells at Various Intervals following Inoculation.*

Activities expressed in µl. of 0·1 M NaOH per 70,000 cells in incubation mixture; time of incubation 2 hours.

Time after tumour inoculation.	Mouse.	Activity in µl. of 0·1 M NaOH. Mean of four determinations. dl-Alanyl-glycine. pH 7·8.	dl-Leucyl-glycine. pH 7·5.	dl-Leucylgly-cylglycine. pH 7·5.	Glycyltryp-tophane. pH 7·5.	dl-Glycyl-glycine. pH 7·5.
3 days	A	0·65	0·25	2·12	1·24	0·30
	B	0·68	0·71	0·95	1·35	—
6 days	C	3·05	1·84	3·10	1·64	1·55
	D	2·10	1·43	2·95	1·21	—
13 days	E	6·55	3·06	4·85	1·04	1·60

The presence of a dipeptidase inhibitor in the ascitic fluid was observed in two series of pooled Krebs 2 tumour cells. This inhibitor could be removed at least to some extent by repeated washings in 2 per cent NaCl solution. By such means the dipeptidase activity of the cells could be increased by 10 to 40 per cent over the original figures obtained on non-washed cells. Maximum removal was reached after two washings for 3 to 5 minutes each. The occurrence of this inhibitor was not further studied. A similar increase in cathepsin activity was not observed following repeated washing.

TABLE II.—*Range of Catheptic Activities without Added Activator.*

Ascites tumour.	Substrate.	Percentage substrate degradation per 10,000 cells and 24 hours.
Ehrlich	Nitrocasein* pH 5·3	0·5–1
Landschütz	Edestin pH 3·8	3 –10
Krebs 2	Nitrocasein* pH 5·3	1 – 2

* Due to insufficient solubility of nitrocasein this substrate could not be used at the catheptic optimum pH 3·8.

At all times after the second day following inoculation, the *catheptic activities* of the three ascites tumours both against nitrocasein and edestin remained at low levels (Table II). Following the addition of cysteine to a final concentration of $2{\cdot}5 \times 10^{-3}$ M/litre in the incubation mixture, the rate of degradation did increase about 3 times. The average catheptic activities calculated per cell showed no significant changes in the course of tumour growth from the third day after inoculation.

COMMENTS.

The increase in average protein content per ascites tumour cell during the 2nd to 8th days after inoculation indicates that protein synthesis predominates during this time in comparison with the later stages of ascites tumour growth. The greater rate of increase in cellular protein content during the first few days of the growth coincides with the period of maximum mitotic frequency (Klein

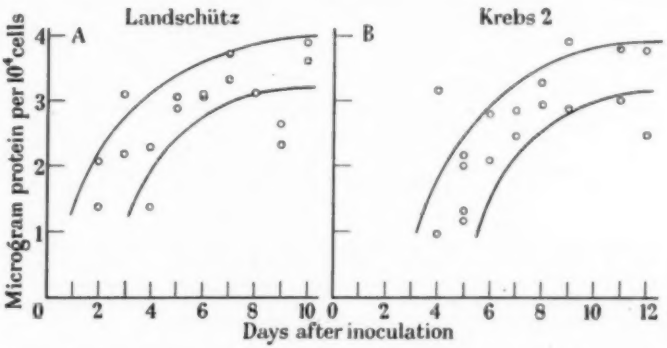

FIG. 1.—Total amount of protein per 10,000 ascites tumour cells during tumour growth. (A) the "Landschütz" variety of Ehrlich; (B) the Krebs 2 ascites tumour.

and Révész, 1953; Lasnitzki, 1953). After the 8th day (Fig. 1) protein synthesis and degradative processes seem to balance each other more or less.

The enzymic data suggest a gradual increase in the average dipeptidase activity per tumour cell with the time after inoculation until a certain level has been reached about the 6th to 8th day after inoculation (Fig. 2). The average dipeptidase activity seems, however, roughly proportional to the protein content of the tumour cells. Similar results have been obtained from studies on other material (cp. review by Linderström-Lang, 1952), indicating high dipeptidase values in various growing cells. On the other hand, the average cathepsin activity per ascites tumour cell (measured without added activator(s)), seems very low and constant during ascites tumour growth.

The observed increase in dipeptidase activity may signify a real increase in enzyme content per tumour cell. It might also be effected by a decrease in the amount of enzyme inhibitor(s) possibly present in the cell material. The presence of such dipeptidase inhibitor has only been demonstrated in the *ascitic fluid*, and could at least in part be removed by washing as mentioned above. Similarly, in

the case of carboxypeptidases, Ballin and Feinstein (1953) described an inhibitor in the ascitic fluid, probably derived from the white blood cells. Our study, however, provides no information regarding available *intracellular* activators and inhibitors.

The ascites tumours are considered as cell populations including a spectrum of tumour cell generations in different stages of growth. In view of this particular heterogeneity, present techniques permit only conclusions as to the enzymic characteristics of the *average* cell population. More specific information on the enzyme content of ascites tumour cells has been obtained by studies on the topical enzymic patterns of solid forms of ascites tumours (cp. Sylvén and Malmgren, 1955).

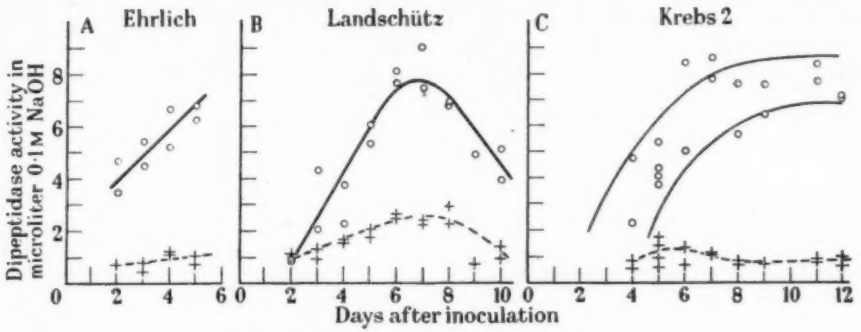

FIG. 2.—Dipeptidase activities against AlaGly during the growth of the Ehrlich, Landschütz and Krebs 2 ascites tumours. Means of four determinations. A and C: Activity per 28,000 cells and 3 hours (upper curves), and calculated per microgramme protein and 3 hours (lower curves). B: Activity per 70,000 cells and 30 minutes (upper curve), and calculated per microgramme protein and 3 hours (lower curve).

SUMMARY.

Serial determinations are reported on the *in vitro* available aerobic catheptic and dipeptidase activities of washed and homogenized Ehrlich, Landschütz and Krebs 2 mouse ascites tumour cell populations at various stages of growth after inoculation. Under present conditions, the total protein content per tumour cell appears to increase until about the 6th to 8th day after inoculation. The average dipeptidase activity measured against *dl*-alanylglycine and calculated per tumour cell shows a similar increase parallel with the time after inoculation and the total tumour cell number. This activity appears to be proportional to the protein content of the tumour cells. During the same time, the average catheptic activity per tumour cell remained low and fairly constant. The proteolytic activities of the ascites tumour fluid, red blood cells and peritoneal cells have been assayed. The presence of a dipeptidase inhibitor in the ascitic fluid is demonstrated.

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STUDIES IN AMINO-ACID UPTAKE BY RD3 SARCOMA CELL SUSPENSIONS *IN VITRO*.

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IN 1913 Van Slyke showed that many cells of the animal body contained free amino-acids at a concentration higher than that in the plasma. Since tissue growth must depend upon the efficiency of capture and concentration of various amino-acids by cells, an understanding of the mechanism for this concentrative uptake might lead to a method by which growth can be controlled. If the mechanism differs appreciably for different tissues, it might well be possible to alter differentially the rate of growth of such tissues. Recently, it has been demonstrated (Wiseman, 1955) that the mono-amino-mono-carboxylic acids compete for the concentrating mechanism of the hamster small intestine and that methionine completely inhibits the active uptake of glycine, L-proline, and L-histidine, when present in equimolecular amounts. When methionine is present at a twentieth the concentration of the proline, the ability of the intestine to concentrate proline is reduced by 50 per cent. Methionine, therefore, should be useful as a cell growth inhibitor by preventing adequate cellular concentrations being attained by some of the amino-acids essential for protein synthesis. In fact, methionine has previously been reported (Pilsum and Berg, 1950; Graham, Hier, Waitkoff, Saper, Bibler and Pentz, 1950; Wretlind and Rose, 1950) as causing retarded growth in rats and the explanation of its action is probably that outlined above.

In their study of the uptake of amino-acids by *in vitro* suspensions of Ehrlich mouse ascites carcinoma cells, Christensen and Riggs (1952) found that the presence of alanine decreased the ability of these cells to concentrate glycine. In an attempt to discover any difference which may exist between the mechanisms in normal and neoplastic cells we have begun a study of the uptake by neoplastic tissue of amino-acids from amino-acid mixtures. Knowledge of such a difference may enable the rate of growth of neoplastic tissue to be suppressed while leaving normal tissue relatively unaffected. The material used was a suspension of cells prepared from a transplantable rat sarcoma and the amino-acids investigated were L-histidine, L-methionine, L-proline, L-lysine and L-ornithine.

It was found that the sarcoma cells in suspension could concentrate against a gradient mono-amino- and diamino-carboxylic acids. The greatest concentration ratio was obtained with L-histidine and the descending order for the others was L-proline, L-ornithine, L-lysine and L-methionine. Generally the amino-acids which could not be well concentrated partially inhibited the cellular uptake of those which could be well concentrated. L-methionine proved to be the best inhibitor of amino-acid uptake and in equimolecular amounts completely prevented L-ornithine and L-lysine from being concentrated by the sarcoma cells.

EXPERIMENTAL.

Tumour.

The RD3 sarcoma used in these experiments had been originally induced by 1:2:5:6-dibenzanthracene injections into the right flank of an inbred strain of albino rats, and has been successfully transplanted subcutaneously in this strain over a period of 20 years (Fig. 1, 2).

Preparation of cell suspension.

Animals bearing a transplanted tumour were killed by a blow on the head and small pieces of tumour tissue from the actively growing periphery of the tumour were excised taking special care to avoid any necrotic areas. These were immediately dropped into about 50 ml. of a bicarbonate-saline (Krebs and Henseleit, 1932) containing 0·3 per cent glucose. The bicarbonate-saline had been previously gassed with 5 per cent CO_2 in 95 per cent O_2 for 20 minutes. This was then vigorously shaken by hand for 5–10 seconds, allowed to stand for about 0·5 minute, centrifuged at 100 r.p.m. for 0·5 minute to remove large fragments, and the supernatant then centrifuged at 1700 r.p.m. for 3 minutes to harvest the free cells from the suspension. The cells were then washed twice with bicarbonate-saline and resuspended. Fig. 3 shows these cells on harvesting. They appeared to be undamaged by the procedure described and remained as single discrete cells. There was some contamination with red blood corpuscles but, as can be seen, the latter can form only a very small proportion by weight of the total cell mass. Further, the ability of the erythrocytes to concentrate amino-acids is relatively poor or non-existent (Christensen, Riggs and Ray, 1952), and hence their presence would not appreciably affect the results. After the last centrifuging the weight of the collected cells was estimated and the amount of bicarbonate-saline used for resuspension was such as to give the required mass of cells per ml. for the particular experiment (varying from 100–300 mg. per ml.).

Amino-acid solutions.

The amino-acids (all of the L-form) were commercial samples of chemically pure grade (purchased from L. Light & Co., Colnbrook, England. Minimum purity 99 per cent.) and were used without further purification. Histidine and lysine were used as the mono-hydrochloride. Ornithine was used as the di-hydrochloride and was half-neutralized by addition of sodium bicarbonate. The amino-acids (singly or in pairs) were dissolved in the bicarbonate-saline (containing 0·3 per cent glucose) to give a 20mM concentration of each amino-acid and the solution was gassed with 5 per cent CO_2 in 95 per cent O_2. On addition of 0·5 ml. of the cell suspension to the amino-acid solution in the Warburg flask the concentration of each amino-acid became 16mM.

General experimental procedure.

0·5 ml. of the sarcoma cell suspension was added to 2 ml. of the appropriate amino-acid solution in the main-chamber of a 25 ml. Warburg flask. The air in the flask was replaced by 5 per cent CO_2 in 95 per cent O_2. The flask and contents were continuously shaken for 1·5 hours at 37° C. in a Warburg bath (rate of shaking 80 oscillations per minute, amplitude 4 cm.). At the end of the experimental period 2 ml. of suspension from each flask was centrifuged at 1700 r.p.m. in tared

tubes for 10 minutes, and the supernatant collected. The tube was carefully dried with filter-paper and weighed, thereby obtaining the wet weight of the cell sample. The cell sample was evenly resuspended in 0·5 ml. of distilled water, the protein precipitated with 5 per cent trichloro-acetic acid, and the filtrate collected. The protein in a measured sample of the supernatant from each flask was also removed by use of 5 per cent trichloro-acetic acid and the filtrate collected. The amino-acid concentration of the initial amino-acid solution was estimated along with that in the filtrate samples obtained from the supernatant and cells of each flask. Control experiments were done using suspensions with no amino-acid in the bicarbonate-saline.

Oxygen uptake of the cell suspension.

The cell suspension was prepared as described above, but a phosphate-saline (Krebs, 1933) containing 0·3 per cent glucose and gassed with O_2 was used to replace the bicarbonate-saline. 100–150 mg. wet weight of cells (in 0·5 ml. suspension) were added to 2 ml. phosphate-saline in the main-chamber of the 25 ml. Warburg flasks. The centre-wells contained KOH insets and the gas phase was O_2. The rate of oxygen uptake during the experimental period of 1·5 hours was determined at both 37° C. and 32° C.

Cell water content.

The cell water content was determined (by drying at 110° C. for 2 hours) in a number of samples from different rats and was found to be 83 per cent. The free amino-acid content found in the cells was assumed to be evenly distributed throughout the cell water and the results calculated in mg./ml. cell water.

Chemical estimations.

Proline, lysine and ornithine were determined by the colorimetric method of Chinard (1952). Histidine was determined by the colorimetric method of Macpherson (1946) and methionine by the colorimetric method of McCarthy and Sullivan (1941).

Standard deviations.

Standard deviations were calculated using the formula for small samples.

RESULTS.

The rate of oxygen uptake by the sarcoma cell suspension at both 37° C. and 32° C. was steady throughout the experimental period of 1·5 hours. At 37° C. the rate was $-$ 4·0 μl. O_2/mg. dry wt./hr. and at 32° C. it was $-$ 2·5 μl. O_2/mg dry wt./hr. This reduction in QO_2 of about 40 per cent for a decrease in temperature of 5° C. is of the order expected for a system where the diffusion of oxygen is not a limiting factor, and as the rate of oxygen uptake was constant during the experimental period, 1·5 hours was chosen as the incubation time.

Table I shows the concentration ratios developed after incubating sarcoma cells in bicarbonate-saline containing single amino-acids or pairs of amino-acids. The concentration ratio is the ratio of the intra-cellular to extra-cellular concentration of the amino-acid at the end of the experimental period. It was found that

when present alone all the amino-acids examined were taken-up against a concentration gradient. L-histidine was concentrated best and the descending order for the others was L-proline, L-ornithine, L-lysine and L-methionine.

TABLE I.—*Amino-acid Concentration Ratios Developed by RD3 Sarcoma Cell Suspensions.*

Concentration ratio is the ratio of the intracellular to extracellular amino-acid concentration. Initial extracellular concentration of each amino-acid 16 mM. Figures shown are mean and standard deviation, with the number of samples in parentheses. Experimental period 1·5 hour. 37° C.

	Concentration ratios developed:				
	when present alone.	in the presence of equimolecular amounts of—			
Amino-acid.		Histidine.	Proline.	Ornithine.	Lysine.	Methionine.
Histidine	3·94±0·48 (15)	—	2·08±0·53 (15)	2·60±0·39 (10)	2·77±0·53 (10)	1·54±0·32 (15)
Proline	2·42±0·22 (15)	1·73±0·31 (15)	—	—	—	1·59±0·08 (18)
Ornithine	2·25±0·05 (10)	1·54±0·07 (10)	—	—	—	1·02±0·08 (10)
Lysine	2·14±0·13 (10)	1·54±0·14 (10)	—	—	—	0·94±0·03 (10)
Methionine	2·12±0·46 (25)	1·47±0·22 (10)	1·69±0·19 (14)	2·74±0·40 (10)	2·00±0·27 (10)	—

When two amino-acids were present in equimolecular amounts each amino-acid generally decreased the ability of the cells to concentrate the accompanying amino-acid. With L-methionine the inhibitory effect was most marked and its presence completely prevented L-ornithine and L-lysine from being taken up against a concentration gradient, while the active uptake of the L-methionine itself was unimpaired by the presence of L-ornithine or L-lysine. Amino-acids which could be only poorly concentrated tended to act as good inhibitors of those amino-acids which could be well concentrated.

DISCUSSION

The rate of oxygen uptake observed at 37° C. (— 4·0 μl./mg. dry wt./hr.) is similar to that quoted by Warburg (1930) for a human sarcoma and Rous sarcoma of chicken (— 5·0 μl./mg. dry wt./hr.) although that for Jensen sarcoma of rat is considerably higher (— 9·0 μl./mg. dry wt./hr.) (Warburg, 1930).

It is interesting to compare the results obtained for the RD3 sarcoma cell suspension with the results obtained by Wiseman (1955) for the hamster small intestine, the only normal tissue on which such a study has been made, although the mechanism for amino-acid uptake by intestine may differ to some degree from the mechanism in other normal cells. The ability of the RD3 sarcoma cells in suspension to take up amino-acids against a concentration gradient is well marked and the mechanism is active for the diamino-acids as well as for the mono-amino-acids. This is in contrast to the findings with hamster small intestine (Wiseman, 1955) which can transfer against a gradient only the mono-amino-acids but not

the diamino-acids. The concentration ratios developed by the sarcoma cells and the hamster small intestine (Wiseman, 1955) are of the same order, although the intestine concentrates proline better than histidine, while the sarcoma cells concentrate histidine better than proline. With both these tissues it was found that poorly concentrated amino-acids act as better inhibitors than amino-acids which are well concentrated. The presence of L-methionine in equimolecular amounts lowered the concentration ratio for L-histidine and L-proline, but did not completely prevent their concentration by the sarcoma cells as it did with hamster small intestine.

The results show a qualitative as well as a quantitative difference between the mechanism in hamster small intestine and the RD3 sarcoma cells, the diamino-acids inhibiting the uptake of the mono-amino-acids in the neoplastic material. It is, therefore, possible that an excess of the diamino-acids in the circulation could inhibit the uptake of some essential mono-amino-acids by these tumour cells without blocking the pathway of these essential acids to normal cells. If such an effect could be produced in the intact animal suppression of tumour growth or its regression might occur. However, the actual inhibitory power of the diamino-acids on the uptake of the mono-amino-acids is small and the technical difficulties of maintaining a high concentration of the diamino-acids in the circulation of tumour-bearing animals over a prolonged period will have to be overcome in order to investigate this possibility.

SUMMARY.

(1) A technique is described for the preparation of a viable suspension of RD3 sarcoma cells from the rat.

(2) These cells on incubation *in vitro* in solutions of amino-acids concentrated intracellularly L-histidine, L-proline, L-ornithine, L-lysine and L-methionine.

(3) Amino-acids which could not be well concentrated tended to inhibit the concentration of amino-acids which could be well concentrated. L-methionine, in equimolecular amounts, completely inhibited the concentration against a gradient of L-ornithine and L-lysine.

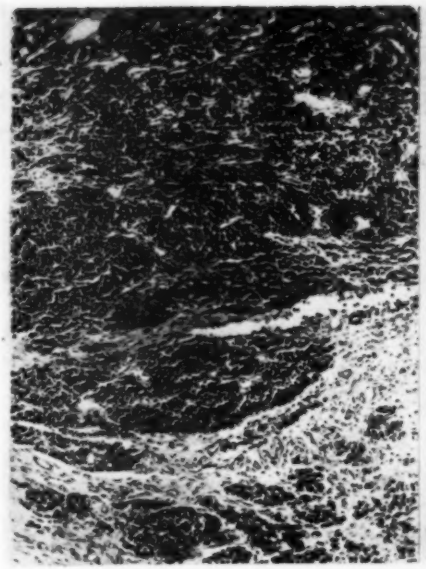
(4) The results indicate that a mechanism exists for active concentration of mono-amino-mono-carboxylic acids and diamino-mono-carboxylic acids and that individual amino-acids compete with each other for this mechanism.

EXPLANATION OF PLATE.

FIG. 1.—Infiltrating edge of sarcoma RD3. H. & E. × 65.

FIG. 2.—High power view of sarcoma RD3 showing a vascular anaplastic tumour composed chiefly of polyhedral cells. H. & E. × 350.

FIG. 3.—Suspension of sarcoma RD3 cells in bicarbonate-saline viewed under phase-contrast. × 320. There is marked variation in size of cells and nuclei, irregular distribution of chromatin, and variation in size and shape of nucleoli characteristic of malignant cells. Free nuclei and nuclear fragments, probably from necrotic and disintegrated tumour cells, are also present. Red blood cells can be discerned lying in the fluid between the tumour cells. Cells in this preparation appear much larger than those in Fig. 2, even though both are viewed at approximately the same magnification. This effect is probably produced by (*a*) shrinkage of cells seen in Fig. 2 produced by the paraffin embedding and H. & E. technique, (*b*) swelling of cells seen in Fig. 3 due to anoxic conditions produced during photographing the preparation, (*c*) variation in size of cells in tumours obtained from different rats.



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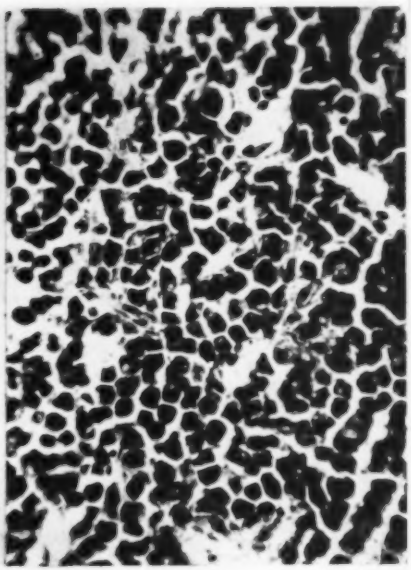

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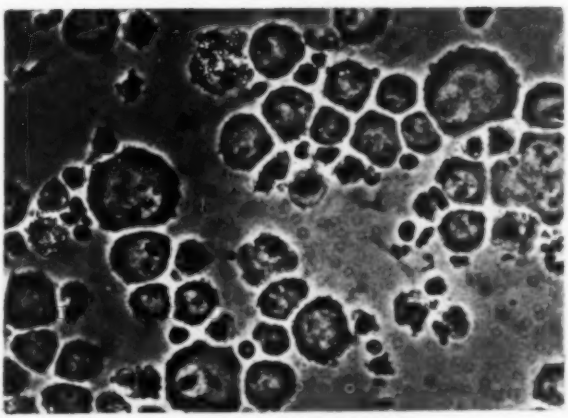

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SURVEY OF PAPERS

TROMP and DIEHL discuss the relationship which they show exists between the incidence of stomach cancer in the Netherlands and certain types of soil (p. 349).

HOCHMAN, RATZKOWSKI and SCHREIBER confirm that the incidence of carcinoma of the cervix in Jewish women is low and conclude that the absence of smegma due to circumcision of Jewish males is the most important factor responsible for this (p. 358).

MORSON compares the incidence of intestinal metaplasia of the gastric mucosa in a series of gastrectomy specimens from cases of gastric ulcer, duodenal ulcer and carcinoma of the stomach. This change he finds most commonly in the pyloric and lesser curvature areas, and most extensive in the carcinoma specimens (p. 365).

MORSON discusses the relationship of intestinal metaplasia of the gastric mucosa and carcinoma of the stomach, and describes five cases in which cancer of the stomach apparently arose in such areas (p. 377).

JOHN and NABARRO find, from a review of the literature and from the study of a series of 347 cases of malignant lymphoma, that intracranial deposits are very uncommon in this group of diseases (p. 386).

WINSHIP and GREENE describe a small series of cases of reticulum cell sarcoma of the thyroid gland, and discuss the difficulties in the diagnosis and treatment of this condition (p. 401).

MARDONES, IGLESIAS and LIPSCHUTZ record the development of granulosa cell tumours in intrasplenic ovarian grafts in two guinea-pigs five years after grafting. They discuss the different types of tumour which may arise in ovarian tissue grafted into the spleen in various species of experimental animal and at varying time intervals (p. 409).

WOODHOUSE reports experiments to determine to what extent and for how long certain carcinogenic and non-carcinogenic polycyclic hydrocarbons are bound to the epidermal proteins of mouse skin after local application. In one group of experiments the application of the hydrocarbon was preceded by treatment with various agents which induce epidermal changes but which are not in themselves carcinogenic (p. 418).

CALCUTT and PAYNE have determined the formation of benzpyrene metabolites in rat liver fractions after repeated doses of the hydrocarbon. They find metabolism ultimately ceases and that there is a loss of lower molecular weight soluble proteins and of lipoproteins (p. 426).

LASNITZKI describes the modification of the effect of 20-methylcholanthrene on mouse prostate grown *in vitro* resulting from the addition of vitamin A to the culture medium (p. 434).

COOPER, GILBERT and LINDSEY find that the hydrocarbons resulting from the burning of cigarette papers are present in far too small quantities to account for the amounts found in the main stream smoke of a cigarette (p. 442).

GWYNN describes the fractionation of croton oil and the isolation of a substance with greatly enhanced tumour-promoting powers (p. 445).

. Berenblum and Haran confirm that urethane, though itself non-carcinogenic for mouse skin, is able to induce in that tissue the initiating phase of carcinogenesis (p. 453).

Schoental and Head report on attempts to produce tumours in mice by the injection of certain methoxy derivatives of polycyclic hydrocarbons (p. 457).

Peacock describes a simple method for injecting cigarette smoke into the thoracic air sacs, and thence throughout the respiratory system of fowls (p. 461).

Finkel and Scribner find that spontaneous tumours of the mammary and reticular tissues are commoner in mice kept in plastic cages than in similar mice kept in metal cages (p. 464).

Malmgren, Sylvén and Révész report serial determinations on the catheptic and dipeptidase activities of the washed and homogenized cell populations of various mouse ascites tumours at various stages of growth after inoculation. They find that the dipeptidase activity per tumour cell increases gradually, apparently proportionally to the protein content of the cell. The catheptic activity per tumour cell remains low and fairly constant (p. 473).

Wiseman and Ghadially, using a suspension of RD3 sarcoma cells from the rat, have investigated the ability of tumour cells to concentrate various amino acids within themselves. They find that amino acids which cannot be well concentrated tend to inhibit the concentration of other amino acids which can be well concentrated (p. 480).